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The Use of Surface Cytokines as Biomarkers in B Cell Mediated Autoimmune Diseases

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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
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The Use of Surface Cytokines as Biomarkers in B Cell Mediated Autoimmune Diseases

by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Microbiology and Molecular Genetics

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABSTRACT OF THE DISSERTATION

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By

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Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, December 2014
Dr. Kimberly J. Payne, Chairperson

RA and SLE are B cell-mediated autoimmune diseases dominated by autoantibodies that affect over 1.5 million Americans. Together RA and SLE contribute to over 29 billion in healthcare costs, therefore due to the high financial burden and physical toll of these diseases on the population, there is a critical need to effectively and efficiently diagnose and treat RA and SLE patients. The aim of our studies was to identify biomarkers and drug targets to improve the identification and treatment of RA and SLE patients. As discussed above, APRIL, BAFF and TSLP have been implicated in the pathogenesis of RA and SLE. Current therapeutics used to treat RA and SLE provide sub-optimal response in a third of patients treated. Therefore, identifying subsets of patients based on their surface and soluble biomarkers and identifying which therapeutics that will provide the most benefit for these patient cohorts has the potential to rationalize the treatment of RA and SLE patients. The cytokine APRIL has been implicated as a potential disease mediator in B cell mediated autoimmune diseases. Our studies show that surface APRIL is expressed on circulating myeloid cells and on total CD19+ B cells and correlates with increased disease in RA patients. Our findings suggest that surface APRIL could provide an easily detectable biomarker and be a useful selection criterion for the

administration of drugs that antagonize APRIL. Another cytokine, TSLP has been implicated in the pathogenesis of RA. We found that TSLP was elevated in the synovial fluid of RA and in the plasma of SLE patients compared to non-inflammatory OA patients indicating that TSLP may act locally in the joints of RA patients and systemically in SLE patients. These studies provide insight into the role of APRIL and TSLP in B cell mediated autoimmune diseases and provide a rationale to guide treatment strategies based upon the expression of APRIL and TSLP. This work is significant because it has the potential to provide clinicians with easily accessible biomarkers to identify the best treatment plan to target these cytokines and their function in pathways involved in RA and SLE pathogenesis.

CHAPTER ONE

INTRODUCTION

The role of the immune system is to detect and destroy foreign antigens while discriminating non-self from self-antigens. Tolerance mechanisms are used to prevent the recognition of self-antigens, however when these mechanisms breakdown autoreactive cells emerge. In autoimmune disease, the detection of autoantibodies, is an indication of the breakdown in negative selection and the presence of autoreactive B cells (1). The development of autoimmune disease is multifaceted with possible genetic and environmental (bacterial or viral infection and smoking) contributions that eventually lead to the breakdown of tolerance mechanisms and the release of autoreactive cells into circulation. The genetic risk factors and environment triggers for the two most common autoimmune diseases—rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) are diagramed in Figure 1 (reviewed in (2-5).

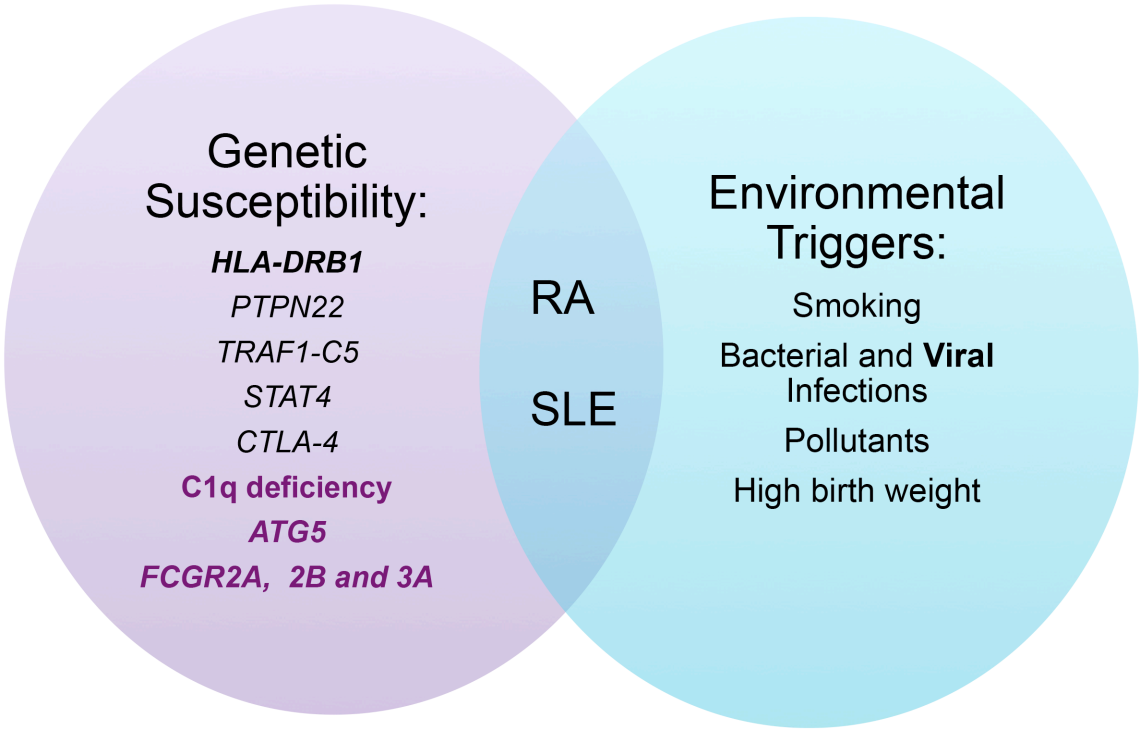

Figure 1: Genetic and Environmental Risk Factors in RA and SLE. RA only risk factors are in black font, SLE only risk factors are in purple font and shared factors are indicated by bolded black font.

B cell Mediated Autoimmune Disease

RA and SLE are B cell-mediated autoimmune diseases dominated by autoantibodies that recognize self-antigens (6, 7). These autoantibodies cause chronic systemic immune responses that result in debilitating pain and multi-organ damage (8). As B cells develop and differentiate into antibody-producing plasma cells, they can be stimulated to proliferate and/or survive at different stages in the process by secreted pro-survival cytokines (9-11). The same factors that propagate the inflammation in autoimmune disease also have the potential to lead to further disease as an increased risk of diffuse large B cell lymphoma is associated with RA and SLE (12, 13).

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic B cell-mediated autoimmune disease dominated by autoantibodies that recognize self-antigens (6, 7). These autoantibodies result in chronic systemic immune responses that target the synovium, cartilage, and bone resulting in joint damage (8). During inflammatory synovitis, immune cells infiltrate the joint (14) and produce cytokines. Many types of immune cells are involved in the joint inflammation including monocytes, dendritic cells (DCs), B cells and T cells and fibroblast like cells within the joint. RA synovial tissue samples reveal an increased frequency of immune cells with T cells having the highest frequency followed by B cells, macrophages and DCs having the lowest frequency (14) suggesting that these immune cells are involved in RA joint damage or they are upstream mediators of the inflammatory cascade that leads to damage. B cells are stimulated by these cytokines at different stages of development to proliferate and differentiate into antibody-producing

plasma cells, thus continuing the cycle of chronic inflammation in RA (9-11). Further evidence of the pathogenic role of these immune cells can be seen in untreated patients diagnosed with early RA who have significantly higher levels of immunoglobulins, autoantibodies and clinical markers of inflammation (CRP and ESR) (15). Disease activity within these early RA patients correlates with immunoglobulin levels and markers of receptor editing further implicating the pathogenic role of B cells in RA (15).

SLE

Systemic lupus erythematous (SLE) is the second most common autoimmune disorder and primarily effects woman of child bearing age (16). In addition, more severe forms of SLE and poorer therapeutic responses are observed in patients of Afro-Caribbean, Native American, Hispanic, and Filipino decent compared to Caucasian patients (17). SLE is characterized by the loss of B cell tolerance and by abnormally activated circulating B cells that produce multiple autoantibodies that target nuclear and to a lesser extent, cytoplasmic antigens (18, 19). These autoantibodies bind to self-antigen inducing inflammation and immune complex formation. Immune complexes ultimately lead to tissue damage when the lodge in the small vessels of the kidney, lung and skin of SLE patients. Abnormal proportions of B cell subsets have been observed in SLE. Within SLE patients, the frequency of transitional 2 (T2) B cells, memory B cells and plasma cells is increased in comparison with healthy donors (20). These patients also have increase serum IgM, IgG, and IgA levels compared to healthy controls likely due to increased plasma cells (21). The development of self-antigens in SLE may be due to the breakdown in cellular mechanisms of removing apoptotic and necrotic cells that leads to

activation of autoreactive B cells. SLE patient have a reduction in monocytes, macrophages and DCs; a reduced phagocytic activity in macrophages, reduced C1q levels production of autoantibodies against C1q, which could result in the inability to remove the source of self-antigens (22-24).

Immune Cells Involved in RA and SLE Pathogenesis

B cells

B cells have been implicated in the pathology of both RA and SLE. B cells contribute through the 1) production of autoantibodies, 2) expression of co-stimulatory molecules, 3) expression of adhesion molecules, 4) antigen presentation, 5) production of chemokines that induce immune cell infiltration, and 6) secretion of cytokines (reviewed in (25, 26).

Normal B cell Development

In the bone marrow, B cells develop from hematopoietic stem cells (HSCs) through a series of sequential steps as shown in Figure 2. Immature B cells emerge from the bone marrow, travel through the periphery, and migrate to the spleen for further maturation and development. During the B cell development process, there are several stages in which B cells are eliminated due to the inability to signal through their B cell receptor or they react strongly to self-antigen. From the pro-B to the pre-B stage, B cells acquire the heavy chain of the B cell receptor (BCR). From the pre-B to immature stage, they acquire light chains. Cells that do not properly rearrange either their light or heavy chain are eliminated. B cells that are autoreactive are removed by negative selection

through apoptosis, receptor editing or anergy during the immature stages of B cell development (27, 28). Immature B cells travel from the bone marrow through the periphery to the spleen where they undergo transitional stages of B cell development. In the spleen, deletion of the autoreactive B cells occurs during the transitional one (T1) and transitional two (T2) stages (29). Surviving T2 cells become either follicular mature (FM) B cells that migrate through the periphery or marginal zone (MZ) B cells that remain static in the spleen. Upon activation, these naïve mature B cells will differentiate into memory B cells or plasma cells (30-32). Surface marker expression allows for the identification of the stages of B cells development. All B cells express the surface marker CD19 and memory cells are identified based on CD27 surface expression. Naïve mature cells are CD19+, CD27-, and IgM+. Naïve mature cells can be further divided into B cells subsets based on the co-expression of CD24 and CD38, which allows for the identification of transitional B cells (CD24++CD38++), mature B cells (CD24+CD38+) and IgM+ memory B cells (CD24+CD38-) (33).

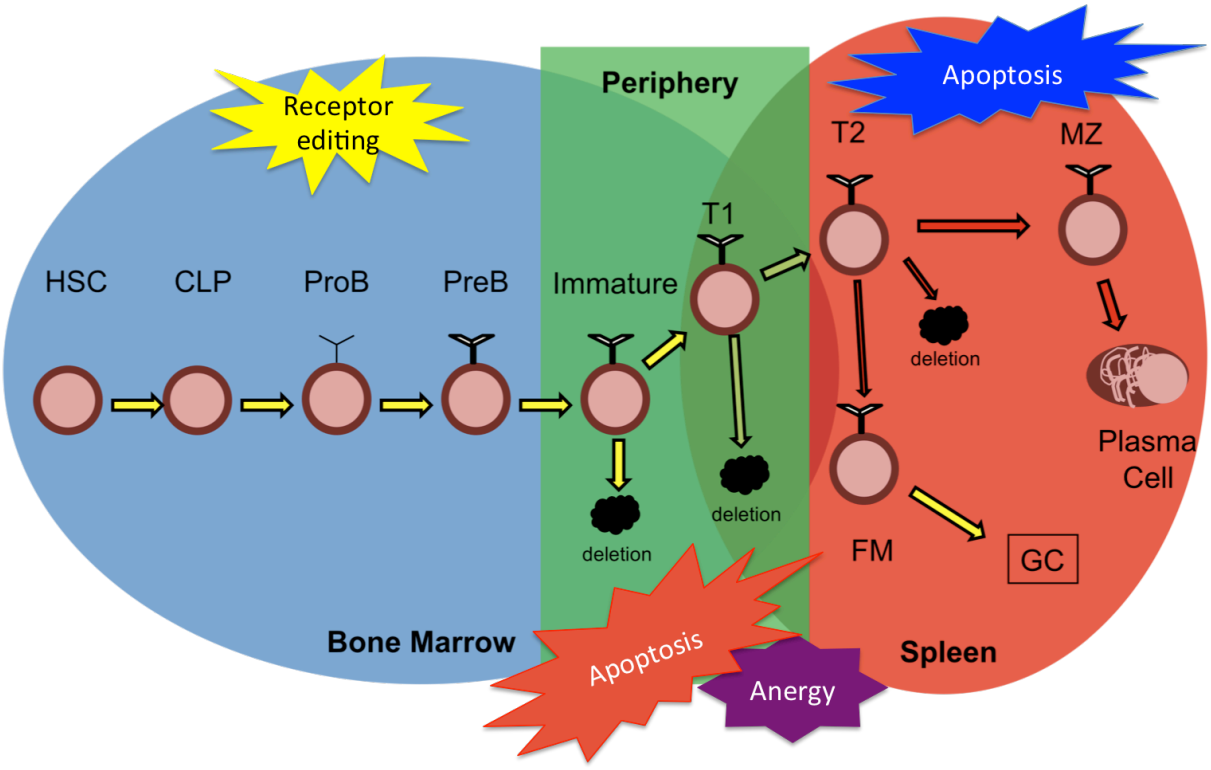

Figure 2: Model of Normal B cell Development. B cells are produced through a series of sequential steps that begins in the BM with hematopoietic stem cells (HSC). Immature B cells emerge from the bone marrow, travel through the periphery to the spleen were these naïve B cells undergo negative selection. It is during the transitional stages (T1 and T2) that autoreactive B cells should be removed by negative selection by receptor editing, anergy or apoptosis. Surviving T2 cells further differentiate into either follicular (circulating) mature or splenic marginal zone B cells. Upon activation, these mature naïve B cells will differentiate into memory or plasma cells.

B cells in Autoimmune Disease

B cells function to make antibodies, present antigens, secrete cytokines and chemokines, and to generate memory cells to enhance the humoral immune response. However, normal B cell functions can be exploited by autoreactive B cells to produce pathogenic autoantibodies, and to cause the downstream activation of additional immune mediators such as T cells. The presence of autoantibodies in both RA and SLE patients indicates a breakdown in negative selection processes. Examination of tolerance checkpoints within SLE patients revealed defects in these checkpoints during the transitional stages of B cell development (see Figure 2) and in germinal centers (GC) allowing autoreactive B cells to differentiate into memory and plasma B cells (34, 35).

The pathogenic role of B cells in RA was first identified with the discovery of rheumatoid factor (RF, auto antibody against constant region of IgG antibodies) and more recently through the use of the B cell depletion therapy Rituximab (monoclonal anti-CD20 antibody) and the identification of anti-cyclic citrullinated peptides (anti-CCP) (26, 36). Unfortunately, current B cell therapies do not directly target plasma cells due to the lack of CD19, CD20, or CD22 expression on these cells, but these long-lived plasma cells eventually die and are unable to be replaced due to continuation of B cell mediated therapies.

While T cells have been associated with RA, recent studies have shown that B cells are required to activate T cells within the synovium (37). Co-stimulatory molecules CD80 and CD86 are highly expressed on B cells from RA and SLE (38). The increased expression of co-stimulatory molecules may increase the antigen presenting capability of these B cells and produce a more efficient T cell activation. Toll-like receptors (TLRs)

also play a role in B cell activation and antibody production in RA and SLE. TLR9 recognizes hypomethylated DNA, and has been shown to increase dsDNA autoantibody production in SLE B cells (39). Immune complexes containing DNA from SLE patient serum can also activate this TLR9 mediated cytokine response in B cells depicted in Figure 3 (40).

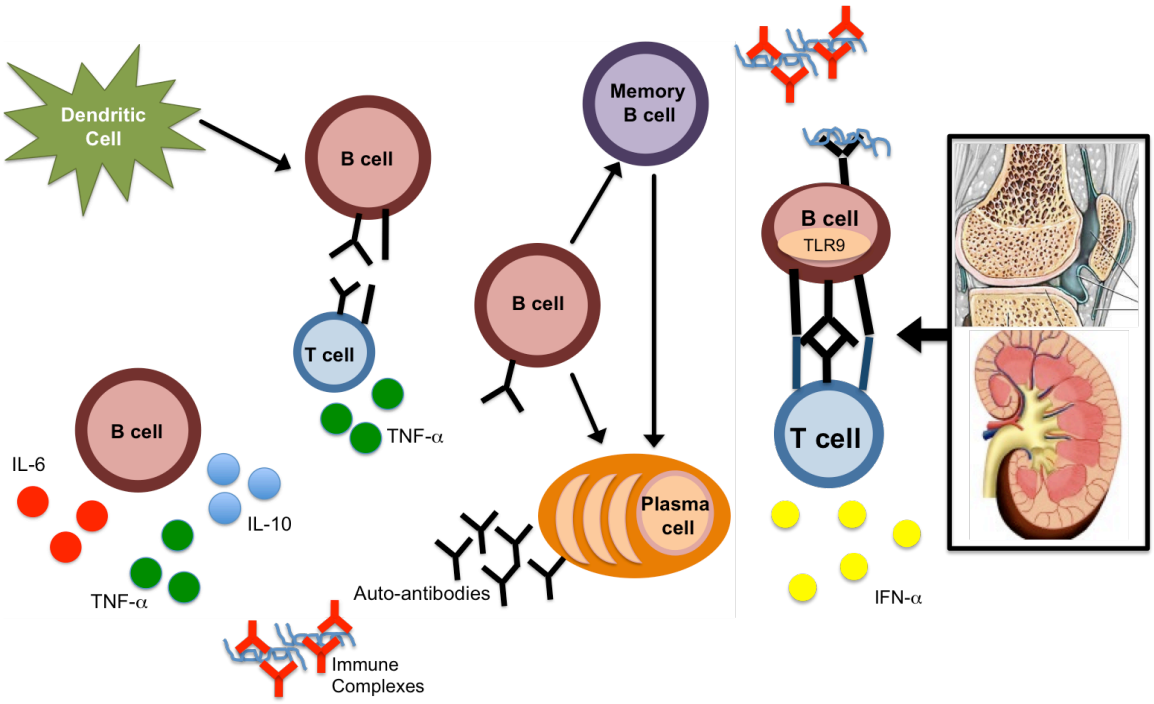

Figure 3: Overview of the Role of B cells in Rheumatoid Arthritis and Systemic Lupus Erythematous. B cells are pathogenic in RA and SLE by producing autoantibodies, presenting antigens, secreting cytokines and chemokines, and by generating memory cells and plasma cells to propagate the autoimmune inflammatory response.

Monocytes

Three monocytes subsets in human peripheral blood have been identified based upon the expression of CD14 and CD16 (FcγRIIIa), and have distinct functional properties. Classical monocytes make up the majority of circulating monocyte population express CD14 but lack CD16 expression. These monocytes specialize in phagocytosis and the production of reactive oxygen species. Intermediate monocytes (CD14+CD16+) express high levels of MHC class II molecules enhancing their antigen presentation capability. Intermediate monocytes also produce the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 when stimulated with LPS. Non-classical monocytes have low-level CD14 and high CD16 expression. These monocytes also produce TNF-α, express MHC class I molecules, exhibit patrolling behavior and are activated by TLR7 and 8 ligands making them important mediators during viral infections (41, 42).

Monocytes that express CD16 (intermediate and non-classical) are considered inflammatory based on their production of inflammatory cytokines and high MHC expression (43). In addition, immune complexes (ICs) from RA and SLE serum can activate FcγRIIIa (CD16) receptors located on monocytes and macrophages leading to the production of TNF-α (44). In RA patients, intermediate monocytes from RA synovium are able to promote the expansion of Th17 cells in vivo, and the frequency of Th17 cells correlated with percentage of CD14+CD16+ monocytes in RA PB (45). In SLE patients, monocytes and macrophages show a reduced phagocytic ability, which could lead to an accumulation of cellular debris and self antigens (23). The pro-inflammatory functions of intermediate and non-classical monocytes implicate them as cellular mediators in autoimmune disease.

Dendritic Cells

Dendritic cells (DCs) are important antigen presenting cells that survey the body for invading pathogens. DCs play an important role in regulating innate and adaptive immune responses through the secretion of pro- or anti-inflammatory cytokines and chemokines that draw immune cells to the area of inflammation. Once DCs encounter antigen, they travel to the lymph nodes where they undergo maturation, upregulate MHC class II and co-stimulatory molecules needed to activate T cells and B cells (14, 46). Two types of DCs have been identified in humans myeloid DC (mDC) and plasmacytoid DCs (pDC) based on surface marker phenotype and their immune functions. mDCs express TLRs 1-6, 8, and 10, while pDCs express TLR7 and 9 (47). pDC also have an additional receptor, CD32, that preferentially binds IgG immune complexes and shuttles these complexes to endosomal location of TLR7 and 9. These TLRs are activated by ssRNA and hypomethylated DNA. SLE and RA patients have elevated levels of circulating CpG motif rich DNA (48), which has the potential to engage these TLRs on DCs and on B cells.

Studies performed on DCs from systemic autoimmune patients indicate that mDC may be more important in the systemic immune response and T cell activation, while pDC response occurs mainly in the site of tissue inflammation and result in B cell activation. Both populations of DCs have been implicated in the pathology of RA and SLE. Increased numbers of both mDCs and pDCs are present in RA patients (49). In the blood and synovial tissues of RA patients, mDCs have elevated levels of MHC class II and co-stimulatory molecules, and are responsible for T cell priming and the production of inflammatory cytokines. pDCs are also found in the synovial tissues and are mainly

responsible for increased IFN-α and BAFF production on B cell activation (50). These DCs produce BAFF and APRIL (cytokines involved in B cell survival and activation) inducing T cell independent B cell production of IgG and IgA antibodies (51). Mouse mDCs can ingest apoptotic blebs, which leads to DC maturation, upregulation of co-stimulatory molecules, production of IL-6 and TNF-α and skewing toward a Th1/Th17 response (52). In SLE patients, mDC have increased activation, induce the differentiation of Th1 and Th17 cells and pDC have been identified in tissue lesions and nephritic kidneys. pDCs are increased in RA patient synovium and correlate with anti-citrullinated peptide antibody titers (50).

Inflammatory Mediators Elevated in RA and SLE

The factors contributing to systemic B cell mediated autoimmune diseases are multifactorial and involve a complex network of immune cells and inflammatory mediators. In our studies, we focus on attention on the inflammatory mediators that influence B cell activation and differentiation and the cells involved in their production and signaling.

TSLP

Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine that signals through the TSLP receptor (TSLP-R). TSLP is primarily produced by epithelial cells in the skin, gastrointestinal tract, thymus, and lungs and by immune cells such as DCs, mast cells, basophils, and fibroblasts (53-57). TSLP induced signaling requires the heterodimeric TSLP-R receptor consisting of an IL-7 receptor alpha (IL-7Rα) subunit

and a TSLP-R subunit, and upon activation, signals through the Jak-STAT pathway in human cells. TSLP-R is expressed on DCs, NK cells, mast cells, and T and B cells (58).

The current paradigm of TSLP's role in inflammation is that it directs a DC mediated differentiation of CD4+ T cells into Th2 cells (59). This mechanism of TSLP activated DCs inducing a Th2 response is seen in asthma, atopic dermatitis, systemic sclerosis, and cancer (56, 60-62). However, recent studies have shown that TSLP may also induce a Th17 response in Hepatitis C infected hepatocytes and in atherosclerosis (60). These studies suggest that the role of TSLP may differ based on cell type. Recently, synovial fibroblast from RA patients have been shown to produce TSLP when stimulated with TLR ligands through a NF-κβ mediated pathway (63). TSLP is further implicated in RA, as it is elevated in the synovial fluid of RA patients and produced a RA-like disease in an RA mouse model (64, 65).

Tumor Necrosis Factor Super Family Members

Members of the tumor necrosis factor superfamily have been implicated in the pathology of multiple autoimmune diseases. Several TNFSF family members have emerged as targets for therapy in the treatment of autoimmune diseases such as SLE. The normal function and role in B cell mediated autoimmune diseases will be discussed below.

BAFF

B cell activating factor (BAFF, Blys, TALL-1, THANK, TNFSF13B and zTNF4) is a type II transmembrane protein, expressed as a cell surface protein, and released in its

soluble form by proteolytic cleavage by furinase. BAFF protein has multiple biologically active forms: membrane-bound and soluble trimers, 20-mers, and 60-mers in humans (66). BAFF is the ligand for three receptors BAFF-R, the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) receptor and the B-cell maturation antigen (BCMA) receptor.

Increased BAFF levels lead to increased numbers of B cells in vivo (67) indicating that BAFF may be responsible for B cell homeostasis. Inflammatory cytokines promotes B cell production of BAFF (68). In vitro studies show, that BAFF increases cell splenic memory B cell survival and recovery following memory B cell activation (69).

BAFF may be more important for mature autoreactive B cell survival than naïve B cells (70) as B cells compete for BAFF for survival and therefore in mice with a diverse B cell repertoire autoreactive B cells are competitively eliminated. Autoimmunity may develop due to the increased threshold of chronic BCR stimulation that is needed to cause negative selection due to elevated BAFF levels (70). Therapeutics that reduce BAFF levels may be beneficial in reducing numbers of autoreactive B cells. Addition of exogenous BAFF to normal and SLE B cell cultures enhanced the cell viability in normal B cells cultures. This could be to the increased occupancy of BAFF-R by BAFF in SLE thereby preventing further activation by exogenous BAFF (71).

BAFF has been implicated in the development and maintenance of RA and SLE through studies in mouse models of lupus and RA as well as clinical studies. BAFF transgenic mice exhibit defects in negative selection of autoreactive transitional T1 cells, elevated numbers of mature B cells and develop SLE-like disease where they produce anti-dsDNA, rheumatoid factor, and anti-nuclear antibodies (11, 72). B and T cells are

increased and the CD4+ cells display a more activated phenotype (73). Th17 cells, a subset of CD4+ T cells are also elevated in mice that overexpress BAFF, and are decreased in BAFF knockout mice (73). Local BAFF expression in the joints of the RA collagen-induced mouse model (CIA) led to increased Th17 cells production and disease activity (74). BAFF knockout mice have a blockade from the T1 to the T2 B cell developmental stage and have reduced autoantibodies (11, 70, 75).

Soluble BAFF is elevated in SLE and lupus nephritis (21, 76-79). Serum levels of BAFF positively correlated with IgG levels in the plasma of SLE patients and with anti-dsDNA titers (21). Soluble BAFF positively correlates with SLEDAI (76), CRP levels (78) and negatively correlates with blood Hb and IgA levels, (78) and BAFF-R expression on CD19+ B cells (76). SLE patients with high disease activity have increased levels of BAFF mRNA in total PBMCs and B cells compared with SLE patients with low disease activity and healthy donors (20, 68, 77, 78). Patients with higher anti-dsDNA antibodies have increased BAFF mRNA expression in CD19+ B cells (20). CD4+ and CD8+ T cells from SLE patients express BAFF mRNA (80). In SLE, BAFF mRNA levels positively correlated with disease activity (SLEDAI), but did not correlate with soluble BAFF (20, 77, 78). However, Lin et al showed that serum BAFF levels positively correlates with BAFF mRNA levels in PBMNCs (21). Elevated BAFF mRNA expression correlates with autoantibody positivity (anti-dsDNA and anti-RNP antibodies) in SLE patients (68). Synovial tissue sections from patients with severe RA have greater BAFF expression than sections from mild RA or OA patients (81). BAFF in SF is higher than in serum in RA patients (82).

Surface BAFF is present on naïve and memory B cells and plasma cells in SLE patients with high disease activity (20). Surface BAFF was identified on CD14+ monocytes within the blood and SF of RA patients (82). Surface BAFF is expressed on leukemic B cells compared to healthy donors (83). TLR9 can induce expression of surface BAFF on human B cells, increased proliferation (84) and can also lead to T cell independent antibody production (85).

APRIL

A proliferation inducing ligand (APRIL, TNFSF13A, TALL-2, TRDL-1) is a member of the tumor necrosis factor super family (TNFSF) that is expressed by monocytes (86), dendritic cells (87), macrophages (88), neutrophils, myelocytes (86), astrocytes (89), adipocytes (90), and activated T and B cells (91). The effects of APRIL are dependent on the receptor that it binds seen in Figure 4. APRIL has two receptors: TACI and BCMA.

Human *APRIL* consists of 6 exons that can be alternatively spliced in to 3 mRNAs to form APRIL β, γ, and δ, and these splice forms are not present in mouse. *In silico* studies suggests that APRIL-β and APRIL-δ isoforms are a membrane bound, uncleavable forms of the APRIL protein (66). In addition, an intergenic splice form exists that is generated from the combination of exons 1-6 of TWEAK (a closely related family member) and exons 2-6 of APRIL producing TWE-PRIL (92). TWE-PRIL has the transmembrane domain of TWEAK and the trimeric form of APRIL as an uncleaveable membrane bound protein (92). Alternative *APRIL* splice forms give rise to surface APRIL (66). BAFF protein is known to be cleaved at the cell surface; therefore, surface

BAFF is a naturally occurring form of this protein (66). In contrast, APRIL was found to be processed intracellulary within the Golgi apparatus and secreted from the cell (93). Surface APRIL has been observed in leukemic B cells, macrophages, and monocytes; however, which isoforms of *APRIL* (or alternatively, TWE-PRIL) led to this surface expression has not been determined (82, 83, 94-96). Two *APRIL* single-nucleotide polymorphisms (SNP) on codons G67R and N96S have been identified (97). The G67R polymorphism is shown to be associated with SLE susceptibility in the Japanese (97), African-American and Hispanic population (98).

APRIL has been implicated in the pathogenesis of RA and SLE. Serum APRIL levels were significantly higher in SLE patients compared to RA patients and healthy donors (99) while another study showed that APRIL was higher in both RA and SLE (79). Discrepancies exist over whether APRIL is higher in the synovial fluid or in the serum of autoimmune patients. Initial reports show that APRIL is higher in the SF than in the serum of RA patients (82). A more recent study shows that serum levels of APRIL were elevated in RA and no difference was seen between APRIL SF samples and normal donors (96). Serum APRIL levels negatively correlate with anti-dsDNA antibody titer in SLE patients (100). Serum APRIL in SLE correlated with British Isles Lupus Assessment Group (BILAG), BILAG musculoskeletal, and BILAG cardiorespiratory score (99). SLE patients with high disease activity have increased levels of APRIL mRNA in B cells compared with healthy donors and these APRIL mRNA levels positively correlated with disease activity determined by SLEDAI (20). Lupus nephritis glomeruli tissues have elevated levels of APRIL and BAFF mRNA compared to healthy donors (101). This could potentially be due to infiltrating inflammatory cells into the kidney of SLE patients.

APRIL and its receptor BCMA are expressed by fibroblast-like synoviocytes (FLS) in RA and not OA synovial tissues. When these cells are treated with exogenous APRIL not BAFF increased APRIL mRNA and inflammatory cytokines IL-6, TNF-α, and IL-1β in RA FLS and not OA FLS. Treatment of RA FLS cells with BCMA-FC ameliorated these effects. APRIL may contribute joint erosion in RA by increasing osteoclast activity through increased expression of RANK-L (96). APRIL is strongly associated with DLBCL in RA and SLE patients with the highest APRIL expression seen in tumors from SLE and RA patients with high disease activity (102). These studies indicate that APRIL is involved in the local and systemic pathology of both RA and SLE and may contribute to further disease development.

BAFF-R

BAFF-R exclusively binds BAFF and is highly expressed on normal human splenic B cells (69) and is present on circulating naïve (CD27-) and resting memory (CD27+CD38-)(71). BCR engagement upregulates BAFF-R expression on T2 and mature B cells (103). BAFF signaling through the BAFF-R increases B cell chemotactic response to the chemokines CCL21, CXCL12, and CXCL13 (104). BAFF induced chemotaxis is mediated through the p38 MAPK pathway (104). This BAFF/BAFF-R function may contribute to the movement of B cells during inflammation as BAFF-R is downregulated in the germinal center B cells (see Figure 4) (105).

Expression patterns and the function of BAFF-R have been examined in RA and SLE patients. No difference in expression of BAFF-R was observed on B cells from SLE patients or from normal donors (71, 80). BAFF-R on CD19+ B cells was reduced

compared to healthy donors and negatively correlates with SLEDAI in patients classified as untreated new-onset SLE (76). However, elevated BAFF-R mRNA expression in total PBMCs positively correlated with SLEDAI scores and anti-dsDNA titers (77). BAFF-R occupancy studies revealed that circulating B cells in SLE patients have consistently occupied BAFF-R which may lead to insensitivity of these cells to BAFF induced activation (71). BAFF-R expression is also increased in synovial tissue from patients with severe RA compared to mild RA and OA patients. This BAFF-R expression was reduced on normal and RA B cells by treatment with NF-kb inhibitor indicating that BAFF engagement of BAFF-R results in the activation of the NF-kb pathway (81).

TACI

The transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) receptor and is a type III class of membrane protein. TACI is the receptor for both BAFF and APRIL as well as heterotrimers of APRIL and BAFF (79). TACI is present of naïve mature B cells and on resting memory B cells (CD27+CD38-) within human spleen (71). The different functions of TACI are shown in Figure 4. TACI signaling has been shown to be responsible for B cell homeostasis and T-independent B cell activation. TACI also stimulates class switch recombination in mature B cells (9).

TACI expression on CD19+ B cells is increased in SLE patients with highest expression in SLE patients with lupus nephritis (76). Glomerular expression of TACI and BCMA mRNA is higher in lupus nephritis (101). Total PBMNC mRNA from SLE patients has higher levels of TACI receptor expression than healthy controls and positively correlates with disease activity (SLEDAI) and anti-dsDNA titers (77). RA and

SLE patients have elevated levels of hypomethylated DNA (106, 107) and increased expression of TLR9 (39). TLR9 stimulation by the hypomethylated DNA increases the expression of TACI and BCMA on human B cells and increases BAFF-R and TACI on murine B cells (84, 108).

BCMA

B cell maturation antigen (BCMA) is expressed on plasmablasts and plasma cells and is responsible for their survival (Figure 4). BCMA is a receptor for both BAFF and APRIL and has been implicated in RA or SLE. Plasmablasts within the normal human spleen express BCMA primarily (71). BCMA is expressed by fibroblast-like synoviocytes in RA synovial tissue and not found in OA tissues (96). Autoantibody producing B cells from SLE patients had higher levels of BCMA compared to BAFF-R compared to normal donors (109).

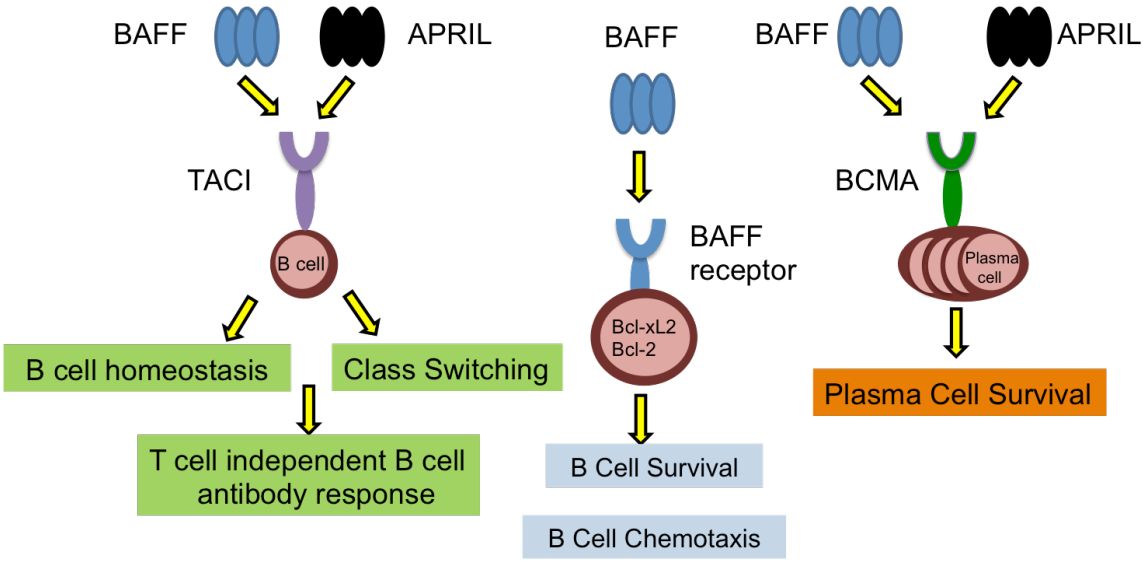

Figure 4: The Role of Secreted BAFF and APRIL Induced Signaling in B cells. The role of secreted APRIL and BAFF is dependent on which receptor these cytokines signal through. BAFF and/or APRIL have been shown to influence B cell survival, activation, chemotaxis, class switch recombination and plasma cell maintenance, all of which could have implications in RA and SLE disease activity. BAFF is the sole ligand for BAFF-R while both APRIL and BAFF bind and signal through TACI and BCMA.

Therapeutics that Target APRIL and BAFF

Disease activity measured by DAS28 in RA patients is reduced following biologic therapy however, inflammatory cells were still present within synovial biopsies from these patients (14) suggesting that therapies that target immune cells over cytokines may be more beneficial for RA patients. T-independent B cell activation through the TNFSF members BAFF and APRIL and TLRs engagement is gaining attention as a potential contributor to autoimmune disease. Corticosteroids are often used as part of the treatment regimen for SLE and soluble BAFF but not APRIL levels decrease with treatment with high dose corticosteroids (100).

Two biologics have been developed that antagonize APRIL and/or BAFF. Belimumab a monoclonal antibody that targets BAFF has recently been approved to treat SLE and is currently in clinical trials for the treatment of RA (110). The implication of APRIL in B-cell mediated autoimmune diseases led to the development of Atacicept, a humanized fusion protein of the Fc portion of IgG and TACI. Atacicept antagonizes both APRIL and BAFF and is currently in Phase III clinical trials for the treatment of SLE (111). APRIL and BAFF have overlapping effects in B cell survival and maintenance, therefore this drug provides promise in targeting multiple autoreactive B cell subsets including plasma cells. In animal models, Atacicept reduces IgM levels, reduces B cell numbers and plasma cells, reduces T-cell independent B cell response s in the spleen, and reduced disease activity but did not affect result in the reduction of memory B cells (112-114). Atacicept has been shown to decrease the viability of activated human B cells and plasmablast and reduce levels of anti-dsDNA antibody production in SLE patients (20). The additional advantage of Atacicept is that it has the ability to neutralize heterotrimers of BAFF and APRIL detected in the serum of SLE and RA patients (79). This suggests

that Atacicept may be more effective treatment than Belimumab for SLE patients with elevated levels of BAFF-APRIL heterotrimers.

Significance

Together RA and SLE contribute to over 29 billion in healthcare costs, therefore due to the high financial burden and physical toll of these diseases on the population, there is a critical need to effectively and efficiently diagnose and treat RA and SLE patients (115). The aim of our studies was to identify biomarkers and drug targets to improve the identification and treatment of RA and SLE patients. As discussed above, APRIL, BAFF and TSLP have been implicated in the pathogenesis of RA and SLE. Therefore we sought to determine the role of these cytokines in our RA and SLE patient cohort. Our central hypothesis is that expression patterns of surface as well as soluble forms of APRIL and BAFF contribute to B cell mediated pathogenesis in RA and SLE and that these expression patterns can be used to determine patient disease activity, and predict response to the biologics that specifically target these cytokines. In addition, we hypothesize that TSLP augments APRIL and BAFF production by DCs contributing to a pro-inflammatory environment locally in the joints of RA patients and systemically in SLE patients. These studies provide insight into the role of APRIL and TSLP in autoimmune cell activity and provide a rationale to guide treatment strategies based upon the expression of APRIL, BAFF, and TSLP. This work is significant because it has the potential to provide clinicians with easily accessible biomarkers to identify the best treatment plan to target these cytokines and their function in pathways involved in RA and SLE pathogenesis.

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CHAPTER TWO

SURFACE APRIL IS ELEVATED ON MYELOID CELLS AND IS ASSOCIATED WITH DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS

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Abstract

Objectives: To assess surface APRIL (CD256) expression by circulating myeloid cells in rheumatoid arthritis (RA) and to determine its relationship to patient disease activity.

Methods: Peripheral blood mononuclear cells (PBMNCs) and plasma were obtained from RA patients and normal donors. PBMNCs were stained for flow cytometry to detect surface APRIL and blood cell markers to identify circulating myeloid cell subsets. Based on CD14 and CD16 phenotypes, monocyte subsets described as classical (CD14+CD16–), intermediate (CD14+CD16+), and non-classical (CD14loCD16+) were identified. Levels of surface APRIL expression were measured by flow cytometry and median fluorescence intensity (MFI) was used for comparisons. Levels of soluble APRIL in the plasma were determined by ELISA. Disease activity was measured by Disease Activity Score out of 28 joints (DAS28).

Results: In RA patients, total myeloid cells showed expression of surface APRIL which correlated with disease activity and with plasma APRIL levels observed in these patients. In normal donors, classical monocytes comprised >80% of circulating monocytes. However, in RA patients, the intermediate and non-classical subsets were elevated and made up the majority of circulating monocytes. In contrast to normal donors, where high levels of surface APRIL were only observed on non-classical monocytes, RA patients showed high levels of surface APRIL expression by all circulating monocyte subsets.

Conclusions: Surface APRIL is elevated on circulating myeloid cells in RA patients where it is highly correlated with disease activity. RA patients also showed skewing of monocytes toward subsets associated with secretion of TNF-α and/or IL-1β.

Abbreviations

RA - Rheumatoid Arthritis

APRIL - A Proliferation Inducing Ligand

TNFSF - Tumor Necrosis Factor Super Family

FLS - Fibroblast-Like Synoviocytes

TACI - Transmembrane Activator, Calcium Modulator, and Cyclophilin Ligand Interactor

BCMA – B cell Maturation Antigen

CD – Cluster of Differentiation

TNF-α - Tumor necrosis factor alpha

IL - Interleukin

TLR - Toll-Like Receptor

DAS28 - Disease Activity Score out of 28 Joints

PB - Peripheral Blood

PBMNCs - Peripheral Blood Mono Nuclear Cells

ELISA – Enzyme Linked Immune Sorbent Assay

MFI – Median Fluorescence Activity

FSC - Forward Scatter

SSC - Side Scatter

BAFF - B cell Activating Factor

VERA – Very Early Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) is a systemic B cell-mediated autoimmune disease dominated by autoantibodies that recognize intra- and extracellular antigens (1,2). These autoantibodies result in chronic systemic immune responses that target the synovium, cartilage, and bone resulting in joint damage (3). During inflammatory synovitis, immune cells infiltrate the joint and produce cytokines (4). Stimulation by cytokines induces B cells at different stages of development to proliferate and differentiate into antibody-producing plasma cells, thus continuing the cycle of chronic inflammation in RA (5-7).

Studies of B-cell mediated autoimmune disease implicate the cytokine, APRIL (A PRoliferation-Inducing Ligand), as a potential disease mediator. APRIL has been shown to support B cell development and survival in mice and humans (5). APRIL is a member of the tumor necrosis factor super family (TNFSF) and is secreted by monocytes (8), dendritic cells (9), macrophages (10), neutrophils, myelocytes (8), astrocytes (11), adipocytes (12), and activated T and B cells (13). Elevated levels of APRIL have been measured in the serum and synovial fluid of RA patients (14,15). In addition, fibroblast-like synoviocytes (FLS) have been shown to secrete APRIL in RA, but not osteoarthritis (16). Novel surface forms of APRIL have recently been reported in human cell lines derived from lymphoid (17) and myeloid malignancies (18). In addition, surface APRIL has been observed by microscopy on synovial macrophages from RA patients (18).

The effects of APRIL are dependent on the receptor that it binds. APRIL has two receptors: 1) TACI (the Transmembrane Activator, Calcium modulator) and cyclophilin ligand Interactor receptor and 2) BCMA (the B-Cell Maturation Antigen receptor). TACI is expressed on B-cells (19) while BCMA expression has been reported on plasma cells

and on FLS from RA patients (16). Binding of APRIL to the TACI or BCMA receptor leads to increased B cell or plasma cell survival, respectively (20).

Monocytes exist as a heterogeneous population in the blood of healthy individuals and three subsets have been identified based on the expression of surface CD14 and CD16. Classical monocytes (CD14+CD16–) comprise the majority (~90%) of circulating monocytes. Intermediate monocytes (CD14+ CD16+) have been described as pro-inflammatory monocytes (21,22). Non-classical monocytes (CD14loCD16+) are also called patrolling monocytes and make up the minority subset in the circulating monocyte pool (23). Classical monocytes are excellent phagocytes and produce IL-6 and IL-8 in response to bacterial pathogens. Intermediate monocytes produce the pro-inflammatory cytokines TNF-α and IL-1β (24). Non-classical monocytes exhibit vascular patrolling activity, poor phagocytic ability, and secrete pro-inflammatory cytokines TNF-α and IL-1 in response to TLR7 and TLR8 stimulation. These non-classical/patrolling monocytes are increased in active RA and are present in the glomerular vessels of lupus patients with lupus nephritis (25-29).

Increases in serum levels of soluble APRIL, and in specific myeloid cell populations, have been associated with RA. A novel surface form of APRIL has been identified and recent studies link its expression to myeloid cells and RA (18). However, expression of surface APRIL by monocyte subsets in normal individuals and its relationship to RA are unknown. In this study, we sought to compare surface APRIL expression on circulating myeloid cells in both normal and autoimmune patients, and to determine whether expression of surface APRIL is related to plasma levels of soluble APRIL and disease activity in RA patients. Here we show that surface APRIL is elevated

on circulating myeloid cells and correlates with disease activity in RA. In normal donors surface APRIL was only observed on non-classical monocytes which make up ~5% of circulating monocytes. In contrast, all monocyte subsets showed high levels of surface APRIL in RA patients. While intermediate and non-classical subsets made up <20% of circulating monocytes in normal donors, they comprised the majority of circulating monocytes in RA patients.

Materials And Methods

Subjects

Subjects were enrolled in this study and gave informed consent under a protocol approved by the Loma Linda University (LLU) Institutional Review Board. All RA patients were from Loma Linda University Division of Rheumatology Clinic, Loma Linda California or Beaver Medical Group Rheumatology Clinic, Redlands, CA USA. All patients had a clinical diagnosis of RA and met the 1987 American College of Rheumatology criteria (30). Disease Activity Score out of 28 joints (DAS28) was used as a measure of RA disease activity (see Table 1). The total patient population consisted of 26 patients (23 females and 3 males) with a mean age of 55.2 ± 15.6 years. Normal peripheral blood (PB) from anonymous adult donors was obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL, USA), and donated by the Blood Processing and Quality Control Lifestream in San Bernardino, CA. Normal plasma was purchased from Tennessee Blood Services, Memphis, TN, USA.

Table 1: Clinical Characteristics of the RA Patient Population

1	M/58	15	pos	0.77	NONE	ELISA, FC
2	F/47	10	pos	1.4	MTX	FC
3	F/57	15	pos	1.53	MTX, Etanercept	FC
4	F/66	24	pos	1.56	LEF	ELISA, FC
5	F/26	12	neg	2.09	Adalimumab	FC
6	F/38	9	neg	2.3	MTX, Rituximab	FC
7	F/61	26	neg	2.3	MTX, Etanercept	FC
8	F/42	11	pos	2.35	Etanercept	ELISA, FC
9	F/64	5	pos	2.35	MTX, HCQ	ELISA, FC
10	F/42	4	neg	2.66	Adalimumab	FC
11	F/62	1	pos	2.72	MTX, PDN	ELISA, FC
12	F/64	6	neg	2.8	MTX, HCQ	FC
13	F/43	27	pos	2.82	MTX, Adalimumab	FC
14	F/56	28	pos	3.34	MTX, PRED	ELISA, FC
15	F/75	15	NR	3.57	MTX, Infliximab	FC
16	F/75	15	NR	3.57	MTX, Infliximab	FC
17	M/46	1	pos	3.7	LEF	ELISA, FC
18	F/47	25	neg	3.8	Naproxen	FC
19	F/21	3	pos	4.35	NONE	ELISA, FC
20	F/33	1	neg	4.58	HCQ	FC
21	F/70	4	neg	4.73	MTX, HCQ	ELISA, FC
22	F/75	5	pos	4.75	MTX	ELISA, FC
23	F/61	6	pos	4.99	MTX, Adalimumab	ELISA, FC
24	F/56	29	pos	5.13	MTX, PRED, LEF	ELISA, FC
25	M/60	4	pos	6.95	NONE	FC
26	F/68	10	NR	NR	MTX	ELISA, FC

NR: not reported, MTX: methotrexate, PRED: prednisone, LEF: leflunomide, HCQ: hydroxychloroquine, ELISA: Enzyme-linked immunosorbent assay, FC: Flow cytometry

Sample Preparation

Blood samples were collected in tubes containing acid citrate dextrose (ACD) from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Whole blood samples were centrifuged at 1500rpm for 15 minutes to remove plasma. Peripheral blood mononuclear cells (PBMNCs) were obtained by red blood cell (RBC) lysis using an ammonium chloride based RBC lysis buffer in both RA patient and normal PB samples.

Soluble Cytokine Quantification

Plasma levels of APRIL were determined by a sandwich ELISA (Human APRIL Platinum ELISA, Bender MedSystems GmbH, Vienna, Austria). ELISAs were performed according to manufacture's instructions on freshly thawed plasma. The ELISA plates were read at 450nm absorbance using the uQuant 96 well plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). A standard curve was run in triplicate for each plate and samples were run in triplicate. APRIL plasma levels were calculated using a linear model in Bio-Tek software KCJunior V1.6 (Winooski, VT, USA).

Flow Cytometry

PBMNCs from RA and normal donors were stained with mAbs in PBS for 30 minutes in the dark at 4ºC. Cells were then washed with PBS and stained with Fixable Viability Dye eFluor® 450 (eBioscience) to distinguish between living and dead cells and incubated for 30 minutes at 4ºC in the dark. Cells were subsequently washed and fixed with a 1% paraformaldehyde solution before analysis using the MACSQuant® Analyzer (Miltenyi Biotec). The following isotype control antibodies were used: mouse IgG1 FITC, mouse IgG1 PE–Cy7 (eBioscience, San Diego, CA, USA); mouse IgG1

APC-Cy7, rat IgG2b PerCP-Cy5.5 (Biolegend, San Diego, CA, USA); mouse IgG1 PE, mouse IgG1 APC (Miltenyl Biotech Inc., Auburn, CA, USA). The anti-human antibodies used were: CD256 PE (clone T3-6), CD66b PerCP-Cy5.5 (clone G10F5), CD1a APC (clone HI149), CD16 APC-Cy7 (clone 3G8) (Biolegend, San Diego, CA, USA); CD14 PE-CY7 (clone 61D3) (eBioscience, San Diego, CA, USA). Compensation beads (anti-mouse Ig k compensation beads, BD Biosciences, San Jose, CA, USA) and cells stained with Fixable Viability Dye eFluor® 450 were used for machine compensation settings. Flow cytometry alignment particles were used as an instrument settings control for each experiment (Sphero™ Ultra Rainbow Fluorescent Particles, Lake Forest, IL, USA). Flow cytometry data analysis was performed using Flowjo data analysis software (TreeStar, Ashland, OR). Relative surface APRIL expression for RA patients was obtained by normalizing the APRIL MFI of each patient sample to the mean APRIL MFI obtained for normal samples (n>11) that were stained with the same antibody combinations and collected with the same instrument settings as the RA patient sample.

Statistical Analysis

Statistical differences were determined using non-parametric Mann-Whitney two –tailed tests using GraphPad PRISM software (GraphPad, San Diego, CA, USA). Correlation analysis was performed using Spearman's one-tailed test. Differences were considered to be statistically significant for $p<0.05$. Grubb's Test was used to exclude significant outliers. The mean and 95% confidence interval is shown in each graph. Asterisks within the figures indicate the following p values: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

Results

Surface APRIL Expression is Elevated on Circulating Myeloid Cells in RA Patients

Multiple blood cells have been shown to produce and secrete APRIL(8-13). Surface APRIL expression has been reported on malignant myeloid cell lines (17) and on macrophages in synovial tissues in RA patients (16,31). However, expression of APRIL by circulating myeloid cells has not been reported. We used multi-color flow cytometry to determine whether surface APRIL is expressed on peripheral blood myeloid cells in RA patients and normal donors. For flow cytometry analysis, intact peripheral blood mononuclear cells (PBMNCs) were gated based on forward (FSC) and side (SSC) light scatter (Figure 5A left panels). These cells were further gated to identify living cells (cells that were negative for viability dye, Figure 5A middle panels). From intact living cells, myeloid cells were identified based on size (indicated by FSC) and granularity (indicated by SSC) (gate shown in Figure 5A right panel).

Surface APRIL was clearly detectable on gated myeloid cells from all RA patients as compared to isotype controls (Figure 5B). In contrast, the majority of normal donor samples showed surface APRIL staining that was similar or slightly higher than isotype controls (Figure 5B). A graph comparing the median fluorescence intensity (MFI) of surface APRIL staining on gated myeloid cells from 16 normal donors and 15 RA patients is shown in Figure 5C. These data show that surface APRIL is elevated on myeloid cells in RA patients as compared to normal controls ($p=<0.0001$).

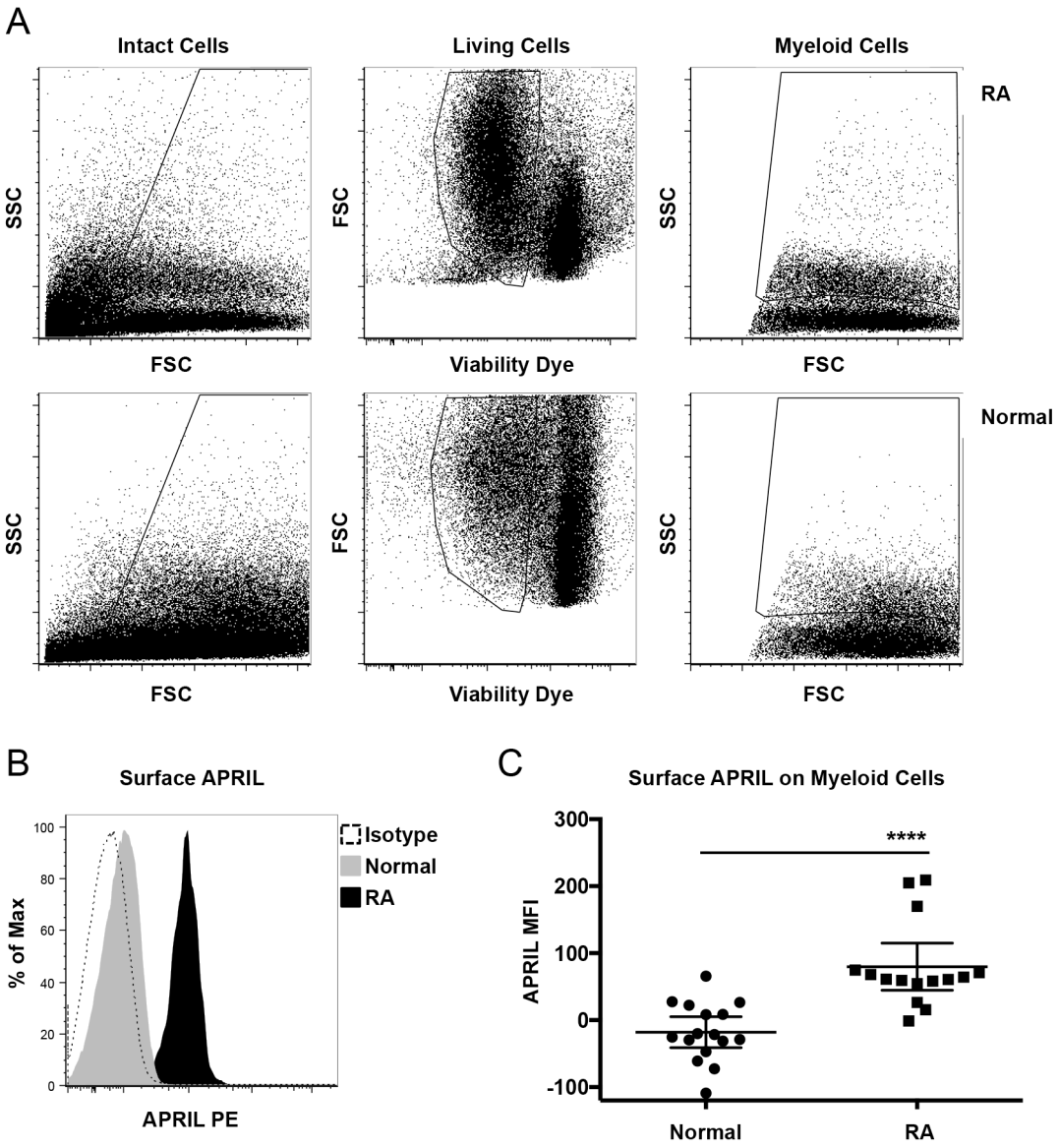

Figure 5. Surface APRIL expression is elevated in RA. A. PBMNCs from normal donors and RA patients were stained for flow cytometry to detect surface APRIL and with viability dye. Forward (FSC) and side (SSC) light scatter and intact cell gate are shown (left panels). From these, living cells were gated (middle panels). Total myeloid cells were gated from living intact cells based on FSC and SSC (right panels). B. Representative histograms show surface APRIL and isotype staining in gated myeloid cells. C. Graphed is the median fluorescence intensity (MFI) of surface APRIL staining on gated myeloid cells from 16 normal donors and 15 RA patients.

Surface APRIL Expression Correlates With Plasma Levels of Soluble APRIL in RA Patients

Elevated levels of soluble APRIL have been reported in the serum of RA patients (16,32,33); therefore, we wanted to determine whether there was a correlation between surface APRIL expression and levels of soluble APRIL circulating in the plasma of RA patients. Plasma APRIL levels were measured in 15 RA patients and 11 normal donors by ELISA. Plasma levels of APRIL were increased in RA patients as compared to normal donors (19.92 ± 25.02 versus 2.12 ± 4.44 ng/mL; Figure 6A). These data confirm previous reports that soluble APRIL is elevated in RA (16,32,33).

Next we evaluated the relationship between surface APRIL expression and plasma levels of soluble APRIL in RA patients. Plasma APRIL levels and surface APRIL expression on myeloid cells (gated as shown in Figure 5A) were determined in RA patients. Patient characteristics are shown in Table 1. Relative surface APRIL expression was calculated by normalizing the APRIL MFI for each patient sample to the mean APRIL MFI obtained from normal donor samples. Spearman's rho analysis shows a positive correlation between relative surface APRIL expression by myeloid cells and plasma APRIL levels in RA patients ($r=0.514$ $p=0.038$; Figure 6B).

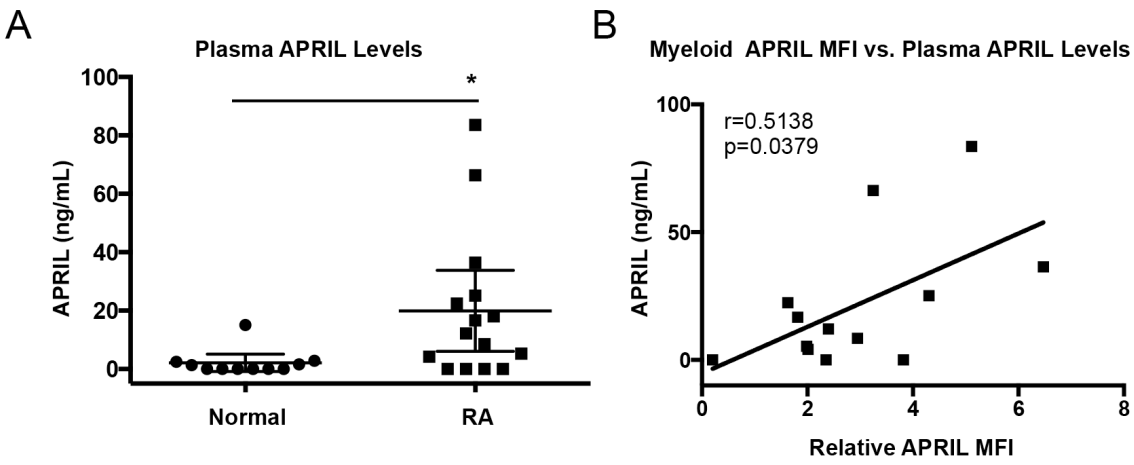

Figure 6: Level of surface APRIL on myeloid cells correlates with soluble APRIL in plasma of RA Patients. A. Soluble APRIL in plasma from normal donors (n=11) and RA patients (n=15) was determined by ELISA. B. Surface APRIL expression by myeloid cells from RA patients was determined by flow cytometry. MFI of surface APRIL staining in RA patients was normalized to that of normal donors to determine the relative APRIL MFI. The Spearman's rho test indicates that relative surface APRIL expression on myeloid cells is highly correlated with plasma APRIL levels in n=13 RA patients.

Surface APRIL Expression Correlates With Increased Disease Activity in RA

Soluble APRIL has been shown to correlate with disease activity in lupus nephritis, systemic lupus erythematous, and juvenile idiopathic arthritis (15,34,35). Increased soluble APRIL levels have been reported in RA patients with active disease (14). Here we evaluated the relationship between surface APRIL expression on myeloid cells and disease activity in RA. The MFI for expression of surface APRIL was calculated as described above. Disease Activity Score in 28 joints (DAS28) found in Table 1, was used to determine the disease activity in our RA patient cohort. In Figure 7, Spearman's rho analysis shows that relative surface APRIL expression by myeloid cells in RA patients positively correlates with DAS28 ($r=0.499$ $p=0.006$). Spearman's rho analysis of soluble APRIL did not show a correlation with disease activity in the RA patients we studied, although their levels of soluble APRIL were elevated as compared to normal donors. These data show that surface APRIL expression by myeloid cells in RA patients correlates with disease activity as indicated by DAS28 score.

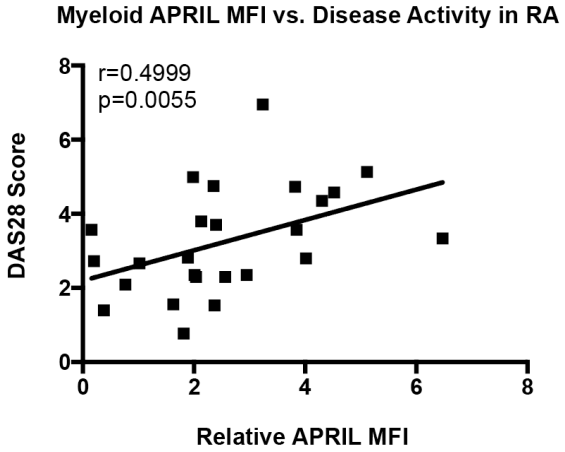

Figure 7: Level of surface APRIL on myeloid cells correlates with disease activity in RA Patients. Surface APRIL expression by myeloid cells from RA patients was determined by flow cytometry. MFI of surface APRIL staining in RA patients was normalized to that of normal donors to determine the relative APRIL MFI. The Spearman's rho test indicates that relative surface APRIL expression on myeloid cells is highly correlated with disease activity as determined by DAS28 in n=25 RA patients.

Frequency Of Intermediate and Non-Classical Monocytes are Increased in the Circulating Monocyte Pool of RA Patients

Given the relationship that we observed between surface APRIL on circulating myeloid cells and disease activity in RA patients, we sought to identify the subsets of myeloid cells responsible for increased surface APRIL expression. Circulating myeloid cells include monocytes as well as very short-lived neutrophils. Previous reports have shown that monocytes are associated with inflammation and that a subset of these monocytes, described as non-classical (CD14loCD16+), is increased in patients with active RA (24,26). Here we used flow cytometry to compare the frequency of classical, intermediate and non-classical monocyte subsets among circulating myeloid cells in normal donors and our RA patient cohort. PBMNC samples were stained with antibodies to detect CD66b, CD14 and CD16. Cells within myeloid light scatter (Figure 8A, left panels) were gated from intact, living PBMNCs as shown in Figure 5A. Monocytes were distinguished from short-lived granulocytes (<1% of living cells in all samples) based on granularity (indicated by side scatter, SSC) and the absence of the granulocyte marker CD66b (gated as shown in Figure 8A, middle panels). The co-expression pattern of CD14 and CD16 was used to phenotypically identify myeloid cells that have been described as classic (CD14+CD16–) intermediate (CD14+CD16+) and non-classical (CD14loCD16+) monocytes (23) (Figure 8A, right panels).

Flow cytometry plots of monocytes in RA samples show a clear increase in CD16 expression that results in a shift from predominantly classical monocytes (CD14+CD16–) to inflammatory intermediate (CD14+CD16+) and non-classical (CD14loCD16+) monocytes. This shift (Figure 8A, right panels) was observed in 12 out of 15 RA patients (The CD14 vs CD16 plots for monocyte subsets in each normal and RA sample are

shown in Figure 10 and Figure 11). A comparison of the frequency of each monocyte subset within the total circulating monocyte pool revealed that the proportion of intermediate and non-classical monocytes were increased (~8 and ~4 fold, respectively), while the proportion of classical monocytes was reduced in RA patients as compared to normal controls (Figure 8B). These data show that the pool of circulating monocytes in RA patients is skewed toward pro-inflammatory, intermediate and non-classical subsets.

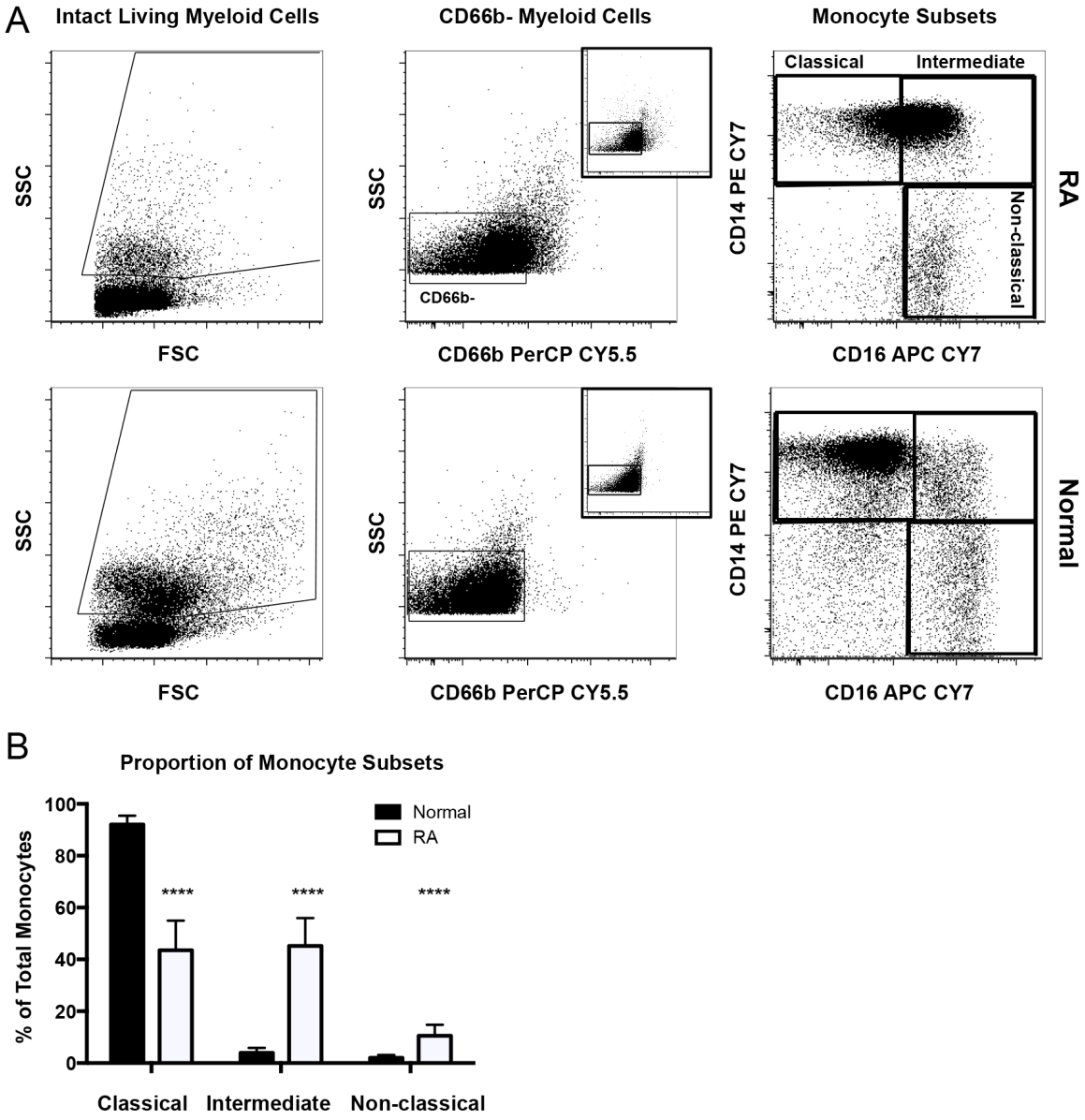

Figure 8. The circulating monocyte pool is skewed toward intermediate and non-classical monocytes in RA. A. PBMNCs from RA patients and normal donors were stained for flow cytometry to detect CD66b, CD14 and CD16. Myeloid cells were gated (left panels) as in Figure 5. Expression of CD66b versus SSC was plotted and monocytes were gated based on low side scatter and absence of CD66b (middle panels.) Co-staining with CD14 and CD16 was used to identify 3 subsets of monocytes (right panels). B. Graphed is the percentage of each subset within the total monocyte pool in normal donors (n=16) and RA patients (n=15).

Surface APRIL Expression is Elevated on PB Monocyte Subsets

Surface APRIL expression has been identified on monocytic leukemia (THP-1) and monocytic lymphoma (U937) cell lines and on primary macrophages from RA patients (16,31). Our data in Figure 5C show surface APRIL expression on some myeloid cells in normal samples, and that this expression is elevated in RA patients. Next we sought to determine normal surface APRIL expression in monocyte subsets and how this compares to RA patients. We evaluated expression of surface APRIL in monocyte subsets gated as described in Figure 5A. Our data show that in RA patients, levels of surface APRIL are elevated in classical and intermediate monocytic subsets as compared to normal donors (Figure 9A-B). APRIL levels were on average increased 3-fold in classic monocytes and increased 6-fold in intermediate monocytes as compared to normal donors. However, there was no significant difference in APRIL expression when non-classical monocytes in RA patients and normal donors were compared (Figure 9C). These data show that in RA patients surface APRIL is upregulated in classical and intermediate monocytes as compared to normal donors.

To gain insights into the potential role of surface APRIL in normal monocytes we compared expression across the classical, intermediate and non-classical subsets (Figure 9D). In normal donors, we found that surface APRIL is undetectable on classical monocytes, expressed at very low levels on intermediate monocytes, and at high levels on non-classical monocytes (Figure 9D). In contrast, RA patients showed similarly high levels of surface APRIL across all monocyte subsets. (Figure 9E). Taken together these data suggest that in RA all monocyte subsets express high levels of surface APRIL normally found only on non-classical monocytes.

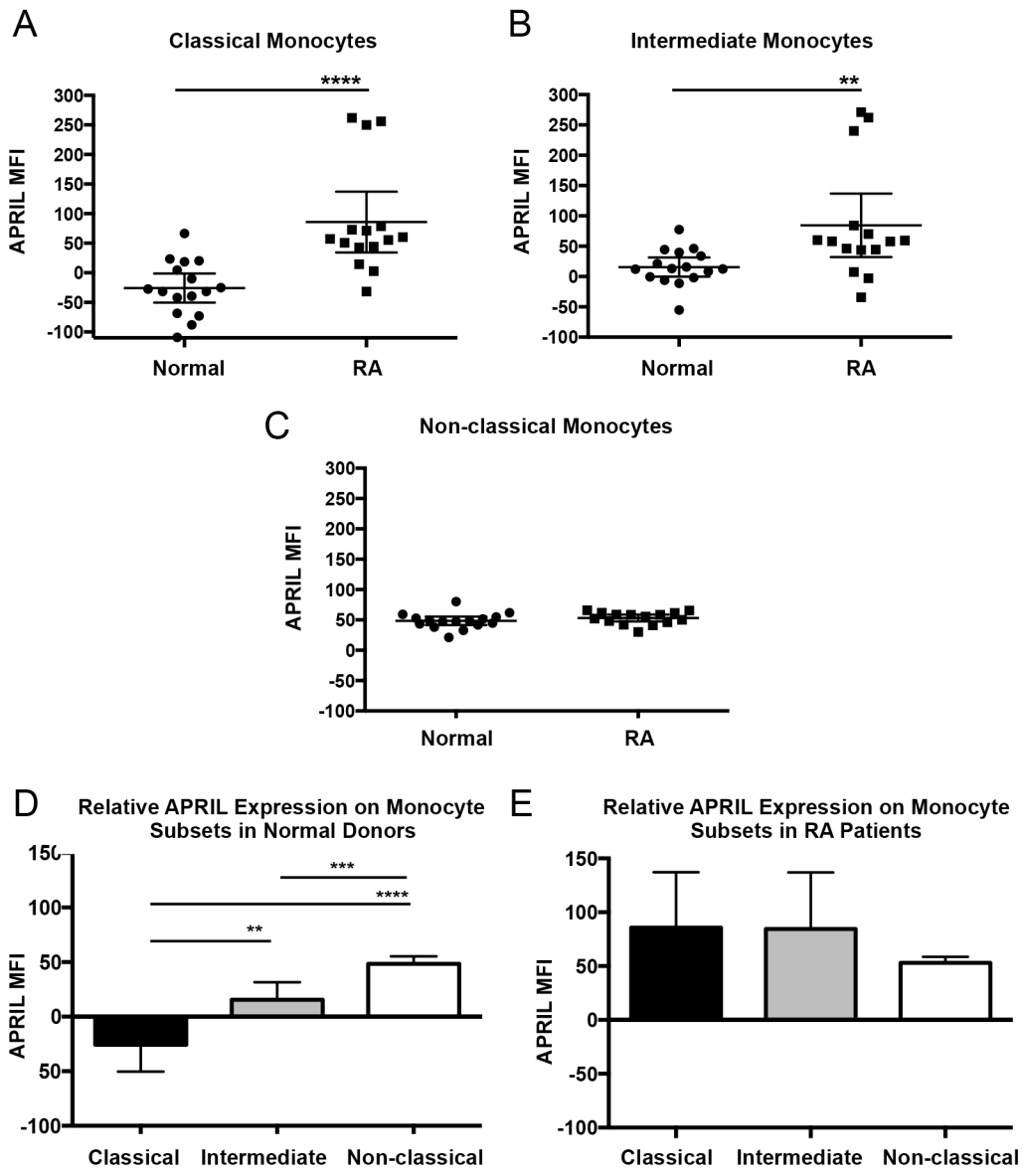

Figure 9. Surface APRIL is elevated on classical and intermediate monocytes in RA patients. PBMNCs from RA patients and normal donors were stained for flow cytometry and monocyte subsets were gated as in Figure 8. The MFI of APRIL staining on monocytes in the (A) classical (B) intermediate, and (C) non-classical subsets in RA patients (n=15) and normal donors (n=16) is plotted. Graphed is a comparison of surface APRIL among monocyte subsets in (D) normal donors (n=16) and (E) RA patients (n=15).

Discussion

Surface forms of APRIL have been identified on cell lines from lymphoid (17) and myeloid malignancies (18) as well as macrophages in the synovial tissue of RA patients (31). Therefore, we sought to determine whether circulating myeloid cells in RA patients express surface APRIL. We show that circulating myeloid cells in RA patients express surface APRIL (Figure 5B and 5C) and this surface APRIL expression positively correlates with disease activity in our RA patient cohort (Figure 7). Thus, our studies are the first to show that surface APRIL is expressed on circulating myeloid cells in RA patients and that levels of surface APRIL are highly correlated with disease activity.

Next we sought to determine which myeloid cell subset(s) are responsible for elevated surface APRIL in RA. Previous reports have shown that the frequency of non-classical monocytes is higher in patients with active RA (26-28). We show that both non-classical and intermediate monocyte subsets are increased in RA patients (Figure 8B). Although these subsets make up less than 20% of the circulating monocyte population in normal donors, in RA patients they represent the majority of circulating monocytes (Figure 8B). Intermediate monocytes are believed to represent a subset of monocytes that are able to rapidly mature and differentiate into tissue macrophages due to their expression of CD16 (36). They also migrate in response to the chemokine CX3CL1 (37), which is elevated in RA patients (38). Thus, the pool of monocytes in RA patients is skewed toward intermediate and non-classical monocytes, populations of monocytes known to produce the inflammatory cytokines TNF-α and/or IL-1β (25), cytokines important in the pathogenesis of RA.

Our evaluation of monocyte subsets from normal donors showed that staining for surface APRIL is not above background in the classical monocytes. Low levels of surface APRIL were observed on intermediate monocytes, while high levels of surface APRIL were only detected on non-classical monocytes in normal donors (Figure 9D). In contrast, surface APRIL is expressed at high levels on all monocyte subsets in RA patients (Figure 9E). Overall, our data suggest that in RA patients a large fraction of monocytes take on features characteristic of the pro-inflammatory monocyte subsets (Supplemental Figure 10 and Figure 11). Specifically, monocytes in RA patients show elevated levels of CD16 (Figure 8A), a feature characteristic of intermediate and non-classical monocytes, as well as high levels of surface APRIL, which in healthy donors was only found on non-classical monocytes (Figure 9D-E.)

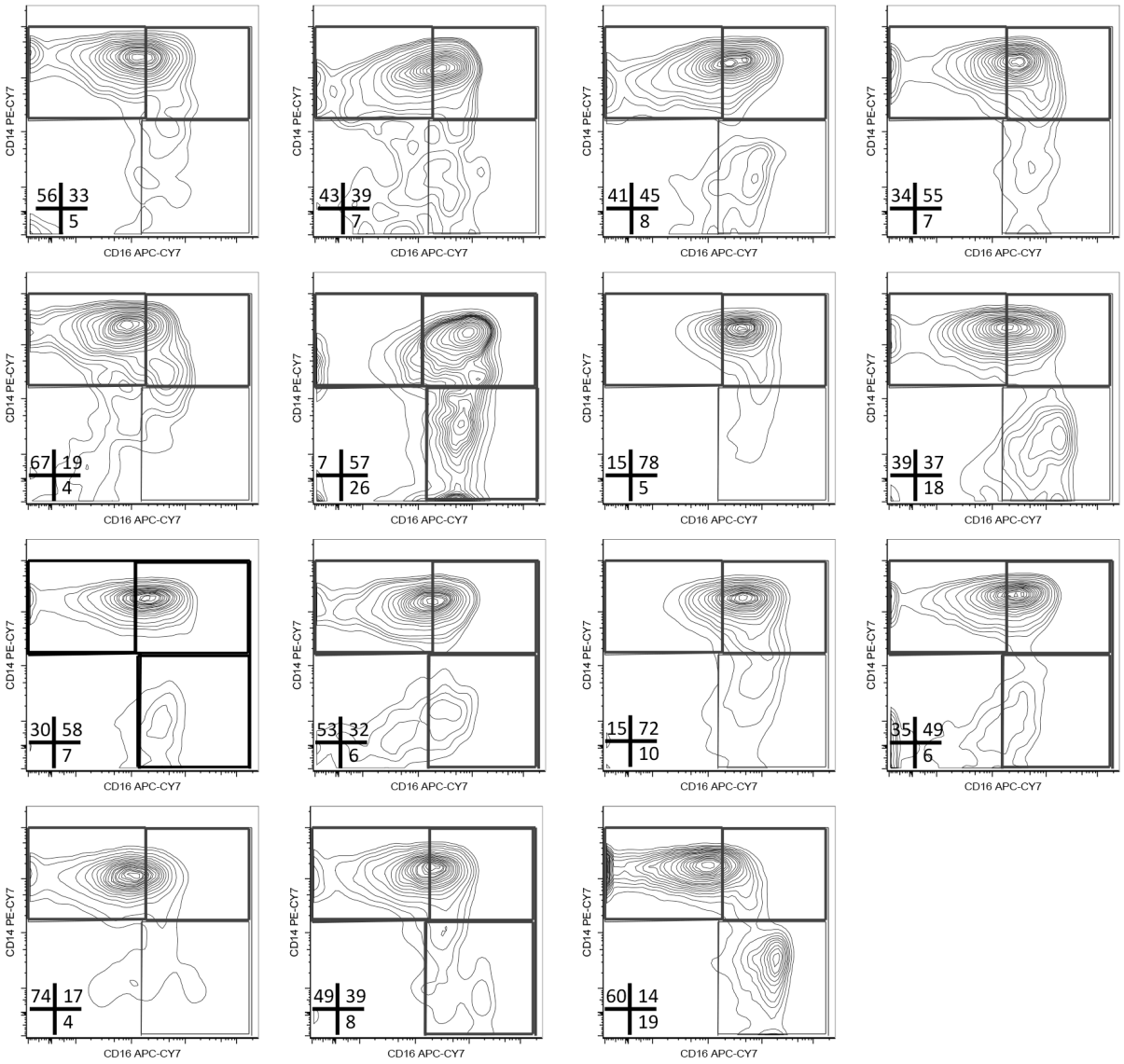

Figure 10. Monocyte subsets in RA patients. Flow cytometry plots showing CD14 versus CD16 co-staining on gated monocytes in PBMNCs from RA patients (n=15). The frequency of gated monocyte for each subset is provided.

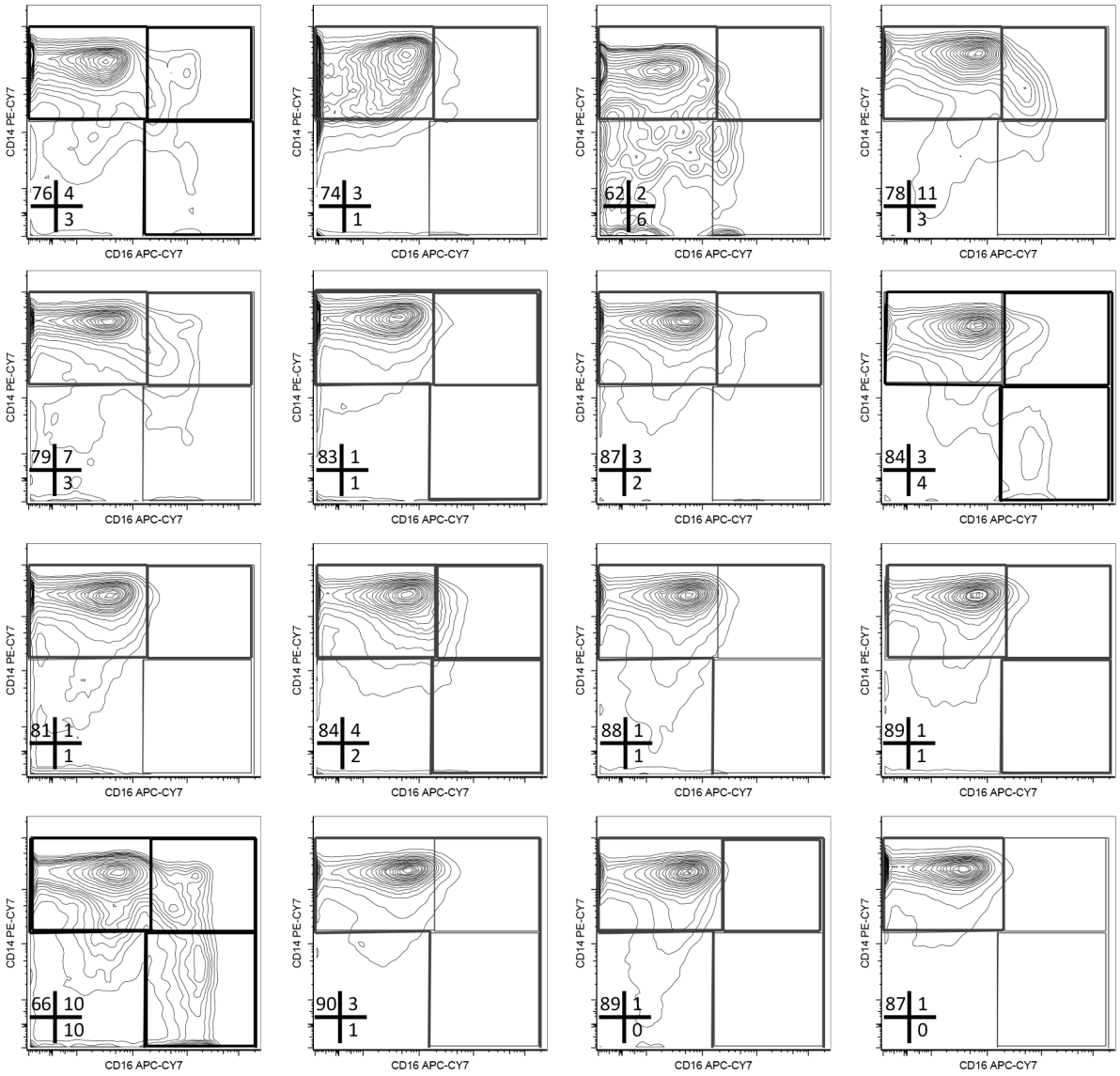

Figure 11. Monocyte subsets in Normal Donors. Flow cytometry plots showing CD14 versus CD16 co-staining on gated monocytes in PBMNCs from normal donors (n=16). The frequency of gated monocyte for each subset is provided.

Surface APRIL has the potential to contribute to RA by acting either as a ligand or as a receptor during interactions with TACI and BCMA. TACI and BCMA are receptors for soluble APRIL. TACI is expressed on normal and malignant B cells, while BCMA is expressed on plasmablasts, plasma cells and on FLS cells in RA joints (16,17,39-43). Cell-to-cell contact between monocytes that express surface APRIL and B lineage cells that express TACI or BCMA have the potential to contribute to RA. It is currently unclear whether surface APRIL gives the same pro-survival signals that soluble APRIL produces in B lineage cells. This will be an important area for future studies.

Surface APRIL has also been shown to act as a signaling receptor when stimulated by interaction with TACI or BCMA. Monocytic leukemia cells expressing surface APRIL produced both IL-8 and MMP-9 when co-cultured with B cells that expressed either TACI or BCMA (18). Since FLS and plasma cells in the RA joint express BCMA, it is possible that cell-to-cell contact results in BCMA-induced surface APRIL signals in myeloid cells and the secretion of pro-inflammatory cytokines that ultimately leads to joint destruction.

Alternative *APRIL* splice forms give rise to surface APRIL (44). APRIL is classically processed intracellularly within the Golgi apparatus and is then secreted from the cell[45]. An intergenic splice form exists that is generated from the combination of exons 1-6 of TWEAK and exons 2-6 of APRIL producing TWE-PRIL (46). The TWE-PRIL splice form has the transmembrane domain of TWEAK and the trimeric form of APRIL as an uncleaveable membrane bound protein (46). TWE-PRIL and other alternative splice forms of APRIL may lead to the surface expression of APRIL (44). Here we show that surface APRIL is elevated on circulating myeloid cells in RA patients.

The function of surface APRIL in the pathology of rheumatic diseases has not been elucidated and will be a focus for future studies.

The implication of APRIL in B-cell mediated autoimmune diseases led to the recent development of Atacicept and other drugs currently under development (47). Atacicept antagonizes APRIL as well as another cytokine, B-cell activating factor (BAFF) and is currently in Phase III clinical trials for the treatment of SLE (48). APRIL and BAFF have overlapping effects in B cell survival and maintenance, therefore, this drug provides promise in targeting multiple autoreactive B cell subsets including plasma cells by preventing APRIL and/or BAFF from activating TACI, BAFF receptor and BCMA. We speculate that Atacicept could also lead to the removal of monocyte populations that aberrantly express surface APRIL.

In two Phase II clinical trials for RA (August I and II), Atacicept reduced RF antibody levels, mature B cells, and plasma cells in patients with inadequate response to either MTX or anti-TNF therapy (49,50). However, these trials failed to meet the primary ACR20-CRP response endpoint (49,50), possibly due to patient selection criteria. Future trials with Atacicept may prove effective in treating patient cohorts with elevated expression of APRIL/BAFF. Data in Figure 7 indicate that ~50% of our RA cohort show moderately elevated surface APRIL, and ~30% have surface APRIL that is more than 3 fold higher than that of normal donors, suggesting that Atacicept may only be effective in a subset of patients.

APRIL and BAFF have been shown to be particularly elevated in very early RA (VERA) suggesting that Atacicept (14,51) may prove beneficial in newly diagnosed RA patients. While all of our patients had established RA, the positive correlation we saw

between plasma APRIL and surface APRIL on myeloid cells suggests that the surface APRIL levels may be even higher in VERA. Our findings suggest that surface APRIL could provide an easily detectable biomarker and be a useful selection criterion for the administration of Atacicept. Surface APRIL expression, together with monocyte subset skewing, might provide a prognostic indicator of RA patient response to Atacicept or other drugs that target APRIL (47).

In conclusion, APRIL is expressed on circulating myeloid cells in RA patients and positively correlates with DAS28 measurement of disease activity. In RA patients, circulating monocytes are skewed toward subsets associated with inflammation and all monocyte subsets show the high levels of surface APRIL normally found only on non-classical monocytes. Further studies are needed to determine the mechanisms responsible for surface APRIL expression and its role in RA pathogenesis.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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CHAPTER THREE

CHARACTERIZATION OF THE SURFACE EXPRESSION OF APRIL AND BAFF AND THEIR RECEPTORS ON B CELLS SUBSETS FROM RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOUS PATIENTS

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Abstract

Background: Autoimmune disease affects more than 23 million Americans. Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are chronic, systemic B cell mediated autoimmune diseases. During normal B cell development, autoreactive B cells are eliminated by negative selection processes that include receptor editing, anergy, or apoptosis. The increased numbers of autoreactive B cells and autoantibodies found in RA and SLE patients suggests that these patients have defects in negative selection that occurs during the immature stages of B cell development. The tumor necrosis factor (TNF) family members APRIL and BAFF have been shown to promote the survival of immature and naïve B cells in mouse models of autoimmune disease. Elevated levels of APRIL and BAFF may contribute to the breakdown of negative selection mechanisms. Serum levels of APRIL and BAFF are elevated in patients with RA and SLE and surface forms of these proteins have been detected on peripheral blood cells from SLE patients and on malignant B lineage cells. Using flow cytometry, we found surface forms of APRIL and BAFF on peripheral blood B cells from RA and SLE patients. The aim of this study was to compare surface expression of APRIL and BAFF and their receptors on B cells from SLE and RA patients to normal B cells and to elucidate their roles in B cell mediated autoimmunity.

Methods: Venous peripheral blood (PB) samples were collected from normal, RA, and SLE patients through an IRB-approved protocol. RA and SLE diagnosis was confirmed using American College of Rheumatology (ACR) criteria. Peripheral blood mononuclear cells (PBMNCs) were separated from whole blood by red blood cell lysis. Human adult peripheral blood samples were stained for seven-color flow cytometry to assess co-expression of CD24, CD21, IgD, IgM, CD38, CD27, CD256 (APRIL), CD257 (BAFF),

CD267 (TACI), and BR3 (BAFF-R). Stained cells were analyzed using a MACSQuant Analyzer (Miltenyi) and FlowJo analysis software (Tree Star). Mean fluorescence intensities (MFI) for surface APRIL and other markers on B cells from RA and SLE patient samples were compared to normal controls by one-tailed, unpaired t-test, $p<0.05$.

Results**:** Peripheral blood B cells obtained from normal (n=11), SLE (n=10), and RA (n=16) were characterized by flow cytometry. The MFI of APRIL, BAFF, BAFF-R and TACI on total CD19+ B cells of RA and SLE patients were compared to normal PB. Surface APRIL expression was significantly higher on B cells from RA and SLE patients as compared to normal donors. The greatest surface ARPIL staining was identified on transitional B cells. BAFF was elevated on RA and SLE CD19+ B cells with greatest expression found on the CD27+ memory B cell subset in RA patients. BAFF-R was elevated on all B cell subsets with greatest expression on transitional B cells in RA patients. TACI receptor, which binds both APRIL and BAFF was also increased on IgM+, CD27+ memory B cells, transitional and mature B cell subsets in RA and SLE patients.

Conclusions: Surface APRIL is expressed on total CD19+ and developmental B cell subsets in RA and SLE patients and positively correlates with disease activity in RA patients. Surface BAFF is elevated on CD19+ and developmental B cell subsets in RA and SLE patients with the highest expression found on CD27+ memory B cells in RA patients. BAFF-R is elevated on B cell subsets in RA patients and negatively correlates with disease activity in SLE patients. In both RA and SLE, TACI receptor is elevated on B cell subsets with the highest expression identified on transitional B cells. The elevated expression of surface APRIL and BAFF on B cell subsets in established RA and SLE

patients suggest that these cytokines may serve as potential biomarkers for the identification of RA and SLE. Further testing is needed to determine how patients with elevated levels of surface APRIL and BAFF respond to targeted therapy by Belimumab and Atacicept.

Abbreviations

RA - Rheumatoid Arthritis

SLE - Systemic Lupus Erythematosus

BAFF - B cell Activating Factor

APRIL - A Proliferation Inducing Ligand

TACI - Transmembrane Activator, Calcium Modulator, and Cyclophilin Ligand Interactor

BCMA - B-cell Maturation Antigen

BAFF-R - B cell Activating Factor Receptor

TNF-α - Tumor Necrosis Factor Alpha

ILX- Interleukin

TNFSF - Tumor Necrosis Factor Super Family

DAS28 - Disease Activity Score out of 28 Joints

SLEDAI – SLE Disease Activity Index

PB - Peripheral Blood

PBMNCs - Peripheral Blood Mono Nuclear Cells

CD – Cluster of Differentiation

MFI – Median Fluorescence Activity

FLS - Fibroblast-Like Synoviocytes

ELISA – Enzyme Linked Immune Sorbent Assay

SSC - Side Scatter

FSC - Forward Scatter

Introduction

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are B cell-mediated autoimmune diseases dominated by autoantibodies that recognize cellular antigens (1, 2). These autoantibodies cause chronic systemic immune responses that result in debilitating pain and multi-organ damage (3). As B cells develop and differentiate into antibody-producing plasma cells, where they can be stimulated to proliferate and/or survive at different stages in the process by secreted BAFF (B cell activating factor) and APRIL (a proliferation inducing ligand) cytokines (4-6).

The effects of APRIL and BAFF are dependent on the receptor that it binds. APRIL has two receptors: 1) the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) receptor and 2) the B-cell maturation antigen (BCMA) receptor. BAFF shares TACI and BCMA with APRIL and has an exclusive receptor BAFF-R (B cell activating factor receptor). BAFF-R and TACI are expressed on B-cells (7) while BCMA expression has been reported on plasma cells and on fibroblast-like synoviocytes (FLS) from RA patients (8). APRIL and BAFF have been shown to influence B cell survival, activation, chemotaxis, and class switch recombination, all of which could have implications in RA and SLE disease activity (9-14).

Consistent with the ability of these cytokines to support B cell development, elevated levels of BAFF and APRIL have been measured in the serum and synovial fluid in RA and SLE (15, 16). APRIL and BAFF are upregulated in response to B cell activation due to dsDNA autoantibody exposure, which is present in both RA and SLE patients (12, 17). These cytokines have been further implicated in B cell-mediated pathologies by the recent identification of surface forms of BAFF and APRIL on leukemic B cells and monocytes, and the report of surface BAFF on B cells from patients

with active SLE (9, 18-22). In synovial fibroblasts, treatment with APRIL leads to the increase of APRIL and the inflammatory cytokines TNF-α, IL-6 and IL-1β (8). Functional analysis of surface APRIL in malignant monocytes showed that stimulation of surface APRIL with soluble TACI induced the production of IL-8 and MMP-9, and suppression of phagocytosis, thereby initiating a pro-inflammatory state (21). Furthermore, BAFF and APRIL have been shown to protect against drug induced cell death through upregulation of the pro-survival molecules BCL-2 and BCL-xL in B cell malignancy (10). These previous studies strengthen the case for the role of APRIL and BAFF in autoimmune disease and suggest that increased levels of these cytokines may be indicators of a poorer prognosis.

The goal of this study was to determine if developmental B cell subsets express surface APRIL and BAFF and if this surface expression correlated with disease activity in RA and SLE patients. In this study, we assessed the surface expression of APRIL and BAFF and their receptors BAFF-R and TACI on circulating B cells and B cells subsets in RA and SLE patients compared to normal donors. In addition, we also assessed their association with disease activity in both RA and SLE. Our data show APRIL is expressed on total CD19+ and developmental B cell subsets in RA and SLE patients and positively correlates with DAS28 measurement of disease activity in RA patients. In addition surface BAFF is elevated on CD19+ and developmental B cell subsets in RA and SLE patients with highest expression found on CD27+ B cells in RA patients. BAFF-R is elevated on B cell subsets in RA patients and negatively correlates with disease activity in SLE patients. In both RA and SLE, TACI receptor was elevated on B cell subsets with the highest expression seen on transitional B cells. Elevated expression of BAFF-R and

TACI on transitional B cells suggests that these cells may be more responsive to APRIL and BAFF signaling. More studies are needed to determine the function of surface APRIL and BAFF in the B cell signaling pathways in RA and SLE, and how patients with elevated levels of surface APRIL and BAFF respond to targeted therapy by Belimumab and Atacicept.

Materials And Methods

Subjects

Subjects were enrolled in this study and gave informed consent under a protocol approved by the Loma Linda University (LLU) Institutional Review Board. All RA and SLE patients were from Loma Linda University Division of Rheumatology Clinic, Loma Linda California or Beaver Medical Group Rheumatology Clinic, Redlands, CA USA. RA patients had a clinical diagnosis and met the 1987 American College of Rheumatology (ACR) criteria (23) and SLE patients met the 1997 ACR criteria for clinical diagnosis (24). Disease Activity Score out of 28 joints (DAS28) was used as a measure of RA disease activity (see Table 2). Systemic lupus erythematosus disease activity index (SLEDAI) was used to assess the disease activity in our SLE patient cohort (see Table 3.) The total patient population consisted of 18 RA patients and 10 SLE patients. Normal peripheral blood (PB) from anonymous adult donors was obtained from Leuko-pak leukocyte filters (Fenwal Laboratories, Lake Zurich, IL, USA), and donated by the Blood Processing and Quality Control Lifestream in San Bernardino, CA.

Sample Preparation

Blood samples were collected in tubes containing acid citrate dextrose (ACD)

from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Whole blood samples were centrifuged at 1500rpm for 15 minutes to remove plasma. Peripheral blood mononuclear cells (PBMNCs) were then isolated by ammonium chloride lysis of red blood cells in both RA patient and normal PB samples.

Flow Cytometry

PBMNCs from RA, SLE, and normal donors were stained with mAbs in PBS for 30 minutes in the dark at 4ºC. Cells were then washed with PBS and stained with Fixable Viability Dye eFluor® 450 (eBioscience) to distinguish between living and dead cells and incubated for 30 minutes at 4ºC in the dark. Cells were subsequently washed and fixed with a 1% paraformaldehyde solution before analysis using the MACSQuant® Analyzer (Miltenyi Biotec). The following isotype control antibodies were used: mouse IgG1 FITC, mouse IgG1 PE–Cy7 (eBioscience, San Diego, CA, USA); mouse IgG1 APC-Cy7, rat IgG2b PerCP-Cy5.5 (Biolegend, San Diego, CA, USA); mouse IgG1 PE, mouse IgG1 APC (Miltenyl Biotech Inc., Auburn, CA, USA). The anti-human antibodies used were: CD256 PE (clone T3-6), CD21 FITC, TACI PE, CD24 PE-Cy7, CD27 APC-Cy7 (Biolegend, San Diego, CA, USA); CD38 PE, IgM PE-Cy5 (BD Biosciences, San Jose, CA, USA); BAFF-R PE (eBioscience, San Diego, CA, USA); CD19 APC (Miltenyi Biotec); and Buffy-2 FITC (Abcam, Cambridge, Massachusetts, USA). Compensation beads (anti-mouse Ig k compensation beads, BD Biosciences, San Jose, CA, USA) and cells stained with Fixable Viability Dye eFluor® 450 were used for machine compensation settings. Flow cytometry alignment particles were used as an instrument settings control for each experiment (Sphero™ Ultra Rainbow Fluorescent Particles,

Lake Forest, IL, USA). Flow cytometry data analysis was performed using Flowjo data analysis software (TreeStar, Ashland, OR).

Statistical Analysis

Statistical differences were determined using non-parametric Mann-Whitney one-tailed tests using GraphPad PRISM software (GraphPad, San Diego, CA, USA). Correlation analysis was performed using Spearman's one-tailed test. Differences were considered to be statistically significant for $p<0.05$. Grubb's Test was used to exclude significant outliers. The mean and 95% confidence interval is shown in each graph.

Results

Identifying B cell Subsets in RA and SLE Patients and Normal Donors

Multi-color flow cytometry was used to assess surface expression of members of the TNF Super Family (APRIL, BAFF, BAFF-R and TACI) on developmental B cell subsets identified based on the gating strategy shown for normal and RA PBMNCs staining (Figure 12A and 12B respectively). For flow cytometry analysis, intact peripheral blood mononuclear cells (PBMNCs) were gated based on forward (FSC) and side (SSC) light scatter (Figure 12A-B panel I). These cells were further gated to identify living cells (cells that were negative for viability dye, Figure 12A-B panels II). From intact living cells, lymphoid cells were identified based on size (indicated by FSC) and granularity (indicated by SSC) (gate shown in Fig.1A-B panel III). B cells were identified based on CD19 expression (Figure 12A-B panel IV) and IgM expression was used to exclude B cell precursors and class-switched B cells (Figure 12A-B panel V).

The human memory B cell marker CD27 was used to distinguish between memory cells from non-memory B cells (Figure 12A-B panel VI). Non-memory B cells were further gated based on the co-expression of CD24 and CD38 to identify transitional and mature B cells subsets in human peripheral blood samples (Figure 12A-B panel VII) (25, 26).

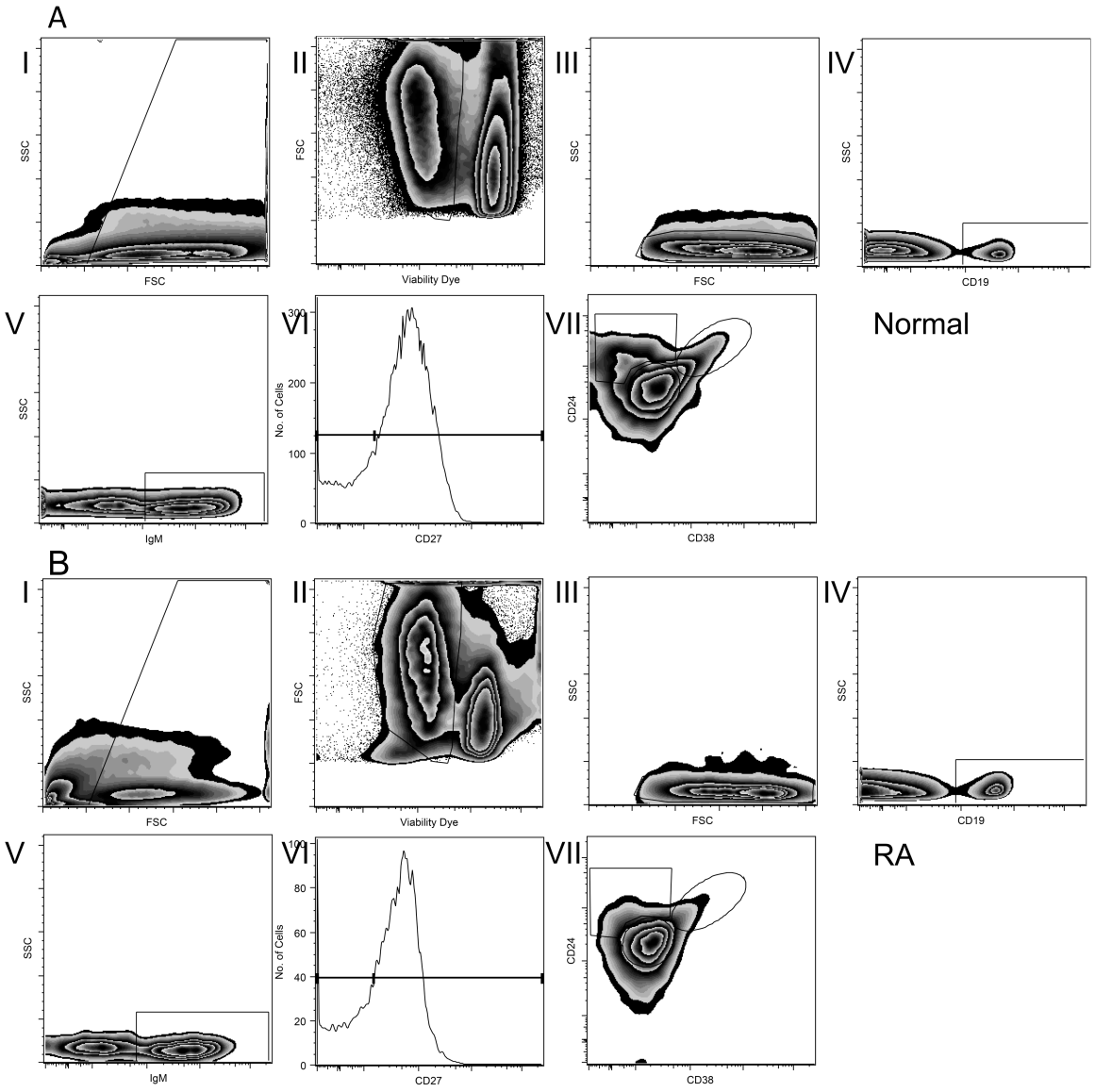

Figure 12. Gating Strategy to Identify B cell Subsets in Normal and RA Peripheral Blood. PBMNC's were stained for flow cytometry to identify B cell subsets in Normal (n=12), RA (n=16) and SLE (n=10) peripheral blood samples. Representative plots are shown for normal (A) and RA (B) PBMNCs. Forward (FSC) and side (SSC) light scatter were used to set a intact cell gate (panel I). From these, living (panel II) lymphoid cells (panel III) were gated. B cells were identified based on CD19+ expression (panel IV). A IgM+ cell gate was used to exclude B cell precursors and class-switched B cells (panel V). Memory and non-memory B cells subsets were identified by CD27 expression as depicted by histogram (panel VI). CD24 and CD38 co-expression was used to identify transitional and mature B cell subsets (panel VII).

Surface APRIL is Elevated on B cell Subsets in RA and SLE Patients

Surface APRIL expression has been identified on leukemic B cells, and is associated B cell survival due to its ability to protect B cells from chemotherapeutic induced apoptosis (19). Our previous studies have shown that surface APRIL is also present on circulating monocytes subsets in RA patients and on non-classical monocytes in normal donors (A. Weldon, et al, Journal of Rheumatology, accepted.) Therefore, we sought to determine if surface APRIL is expressed on developmental B cell subsets from patients with the B cell mediated autoimmune diseases--RA and SLE. PBMNCs from RA and SLE patients were stained to identify B cell subsets as gated in Figure 12. The median fluorescence intensity (MFI) of surface APRIL staining on gated B cell subsets (gated as shown in Figure 12A and B) from 12 normal donors, 16 RA and 10 SLE patients is shown in Figure 13A-E. Surface APRIL is elevated on total CD19+ B cells in both RA and SLE patients. Next, we sought to determine which B cell subsets have surface APRIL expression. Surface APRIL is elevated on memory (CD27+) and immature IgM+ B cells, and on transitional and mature B cells (identified by their CD24 vs. CD38 expression patterns) in RA and SLE patients compared to normal donors. The highest level of APRIL expression was identified on transitional B cells (Figure 13D) in RA and SLE patients. These data indicate that surface APRIL is expressed on B cells from RA and SLE peripheral blood.

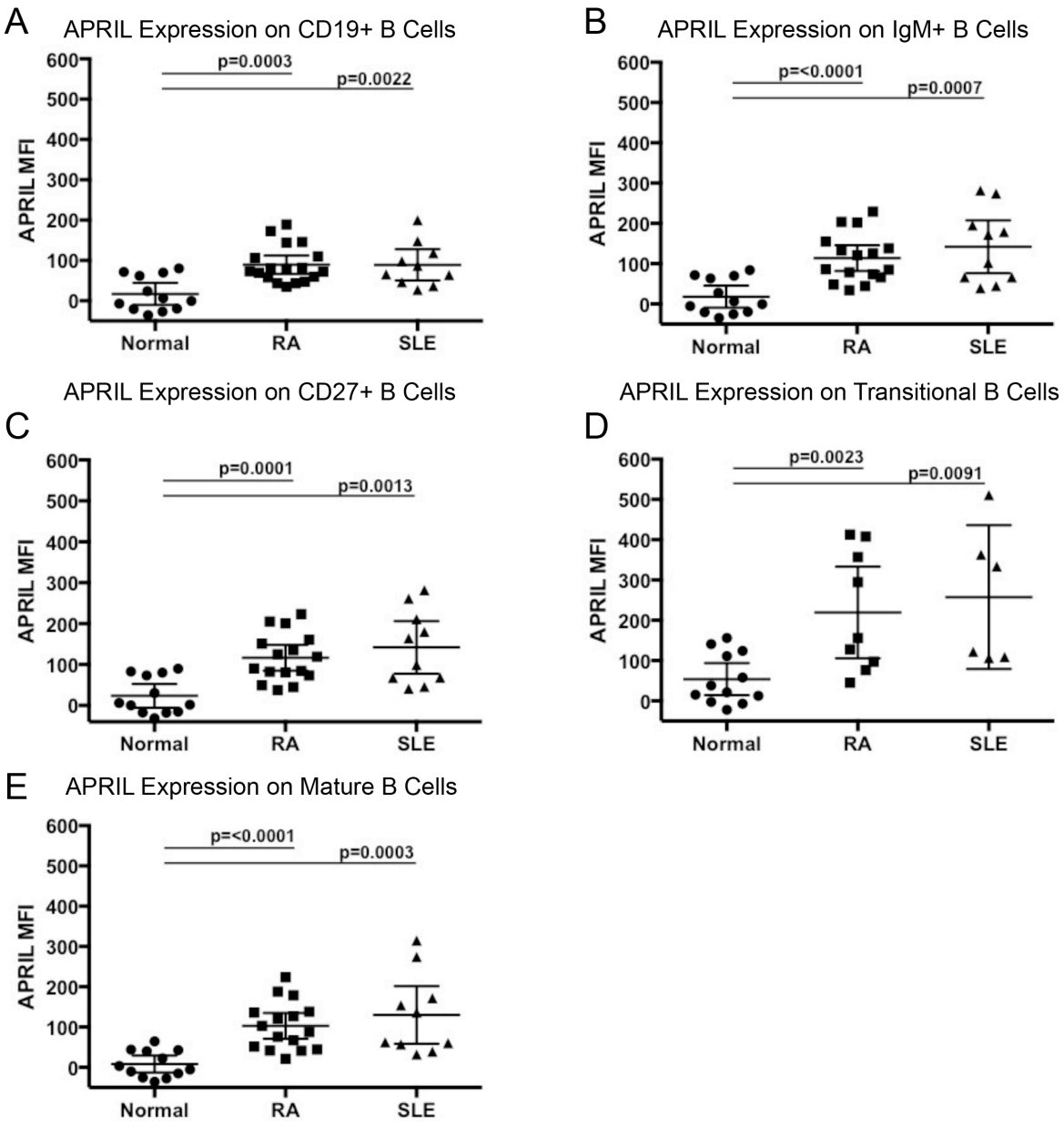

Figure 13. **Surface APRIL is Elevated on B cells from RA and SLE Patients.** Graphed is the median fluorescence intensity (MFI) of APRIL staining on CD19+ B cells (A), CD19+IgM+ B cells (B), and CD19+IgM+27+ memory B cells (C). APRIL MFI on CD19+IgM+CD27- B cells using CD24 and CD38 co-expression patterns identified transitional B cells (D), and mature (E). Samples with insufficient cell numbers were excluded from the graph (D and F). Statistical differences between RA and SLE patients and normal donors were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

Surface APRIL Expression Correlates with Increased Disease Activity in RA

Elevated levels of APRIL have been identified in the serum of SLE and RA patients, and in the synovial fluid of RA patients (8, 27-29). Increased serum levels of APRIL are associated with disease activity in SLE patients (16) and in juvenile idiopathic arthritis (30). RA patients with active disease have increased soluble APRIL levels (15). Recently, our own work showed that surface APRIL on myeloid cells correlates with disease activity in RA patients (A. Weldon, et al. J. Rheumatology, accepted). Here we evaluate the relationship between surface APRIL expression on total CD19+ B cells and disease activity in RA and SLE patients. The MFI for surface APRIL expression on total CD19+ B cells from RA patients (gated as shown in Figure 12B) was determined by flow cytometry. Disease Activity Score in 28 joints (DAS28) found in Table 2, was used to determine the disease activity for each RA patient. The disease activity of our SLE patient cohort was determined by the SLE Disease Activity Index (SLEDAI) and is found in Table 3. In Figure 14, surface APRIL expression on CD19+ B cells shows a positive correlation with DAS28 score ($r=0.4300$ $p=0.0486$). Spearman's rho analysis of surface APRIL expression on CD19+ B cells with disease activity in SLE patients yielded no correlation. This data shows that surface APRIL expression by CD19+ B cells in RA patients is associated with disease activity as indicated by DAS28 score.

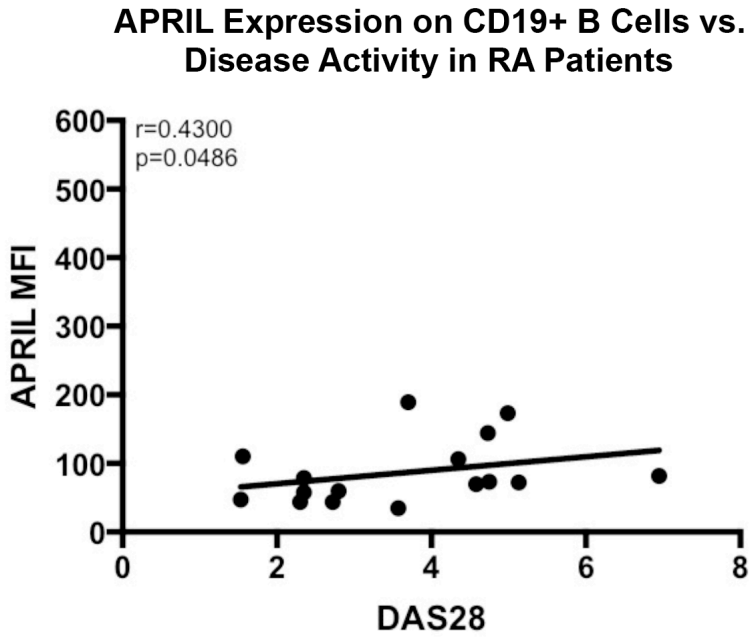

Figure 14: Level of Surface APRIL on CD19+ B cells Correlates with Disease Activity in RA Patients. Surface APRIL expression (MFI) by CD19+ B cells from RA patients was determined by flow cytometry. The Spearman's rho test indicates that surface APRIL expression on CD19+ B cells is correlated with disease activity as determined by DAS28 in n=16 RA patients.

Surface BAFF is Elevated on B cell Subsets in RA and SLE Patients

Naïve, memory, and plasma B cells from SLE patients with high disease activity have elevated surface BAFF expression compared to patients with low disease activity and healthy donors (18). We sought to determine if surface BAFF expression was also elevated on RA B cells and to determine which B cells subsets express surface BAFF. Surface BAFF expression was determined by flow cytometry utilizing the Buffy-2 monoclonal antibody that has been shown not to recognize receptor bound BAFF (9). RA and SLE patients had elevated levels of surface BAFF on all B cells subsets compared to normal donors (Figure 15 A-E). RA CD19+IgM+CD27+ memory B cells had the highest surface BAFF expression (Figure 15C).

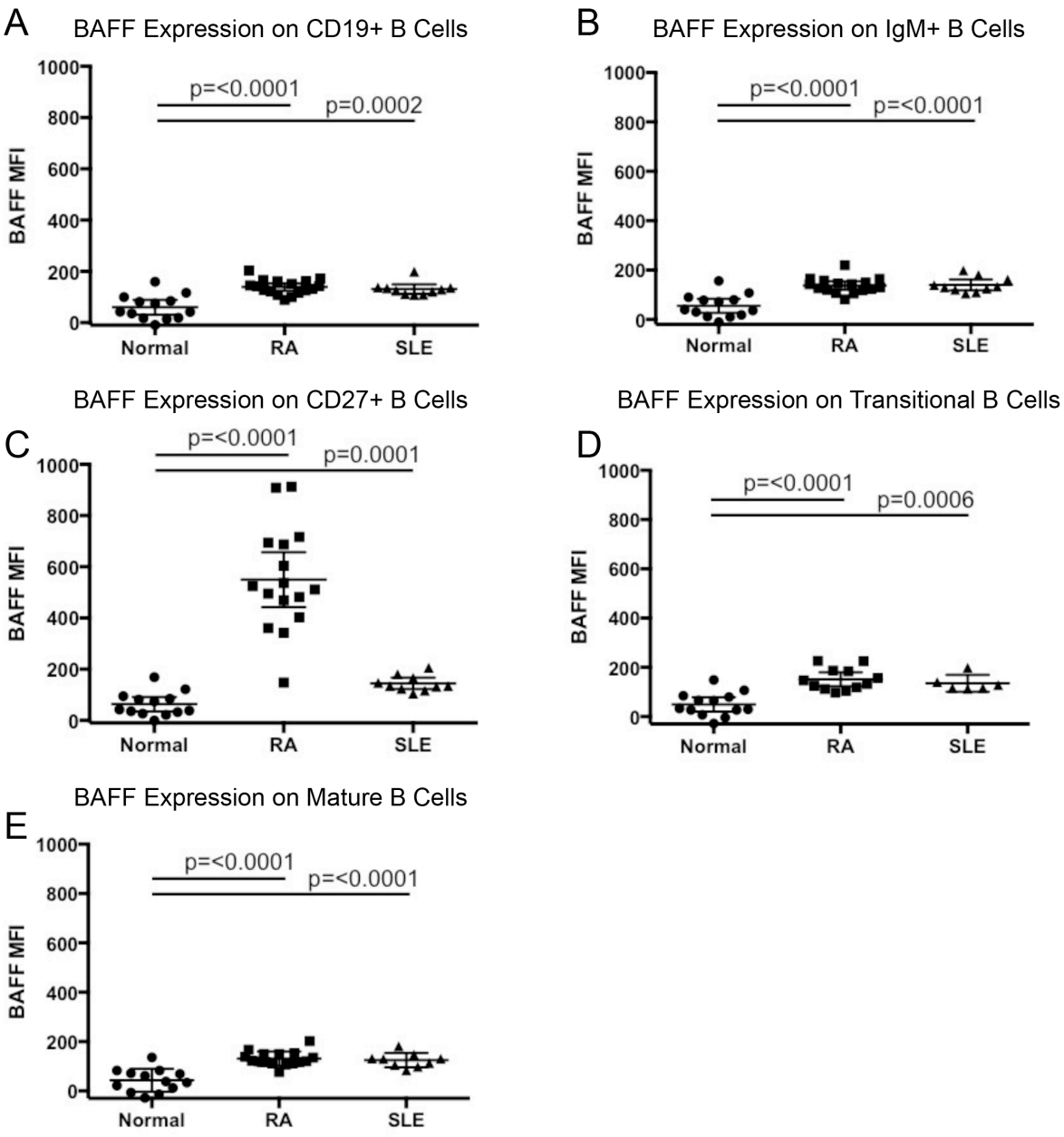

Figure 15. **Surface BAFF is Elevated on B cells from RA and SLE Patients.** Graphed is the median fluorescence intensity (MFI) of BAFF staining on CD19+ B cells (A), CD19+IgM+ B cells (B), and CD19+IgM+27+ memory B cells (C). BAFF expression on CD19+IgM+CD27- B cells using CD24 and CD38 co-expression patterns identified transitional B cells (D) and mature B cells (E). Samples with insufficient cell numbers were excluded from the graph (D and F). Statistical differences between RA and SLE patients and normal donors were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

BAFF-R is Elevated on IgM+ B cells Subsets in RA Patients

Decreased BAFF-R expression on CD19+ B cells from Chinese SLE patients has been shown (31). Due to this finding, we sought to determine the level of BAFF-R expression in our SLE patient cohort and in RA patients compared to normal donors. Living CD19+ B cells were gated in normal, RA, and SLE patient samples as shown in Figure 12. Our data shows no difference in BAFF-R expression on total CD19+ B cells (Figure 16A). However, BAFF-R is elevated on IgM+ B cells (Figure 15B), IgM+CD27+ memory B cells (Figure 16C), transitional (Figure 16D), and mature B cells (Figure 16E) from RA patients compared to healthy donors. No difference in BAFF-R expression was observed on any B cell subsets between our SLE patient cohort and normal donors.

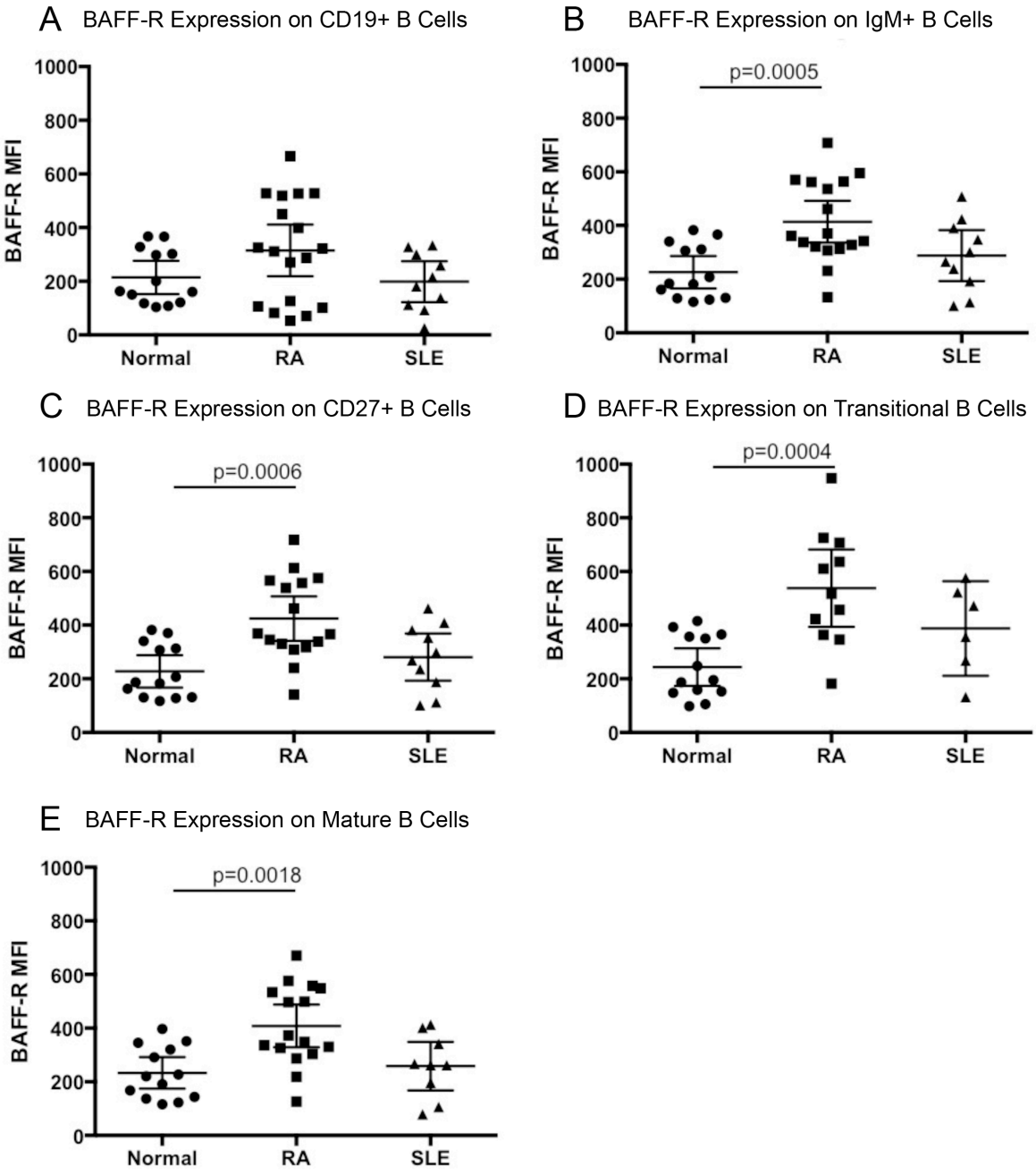

Figure 16. **BAFF-R is Elevated on IgM+ B cells from RA and SLE Patients.** Graphed is the median fluorescence intensity (MFI) of BAFF-R staining on CD19+ B cells (A), CD19+IgM+ B cells (B), and CD19+IgM+27+ memory B cells (C). BAFF-R expression on CD19+IgM+CD27- B cells using CD24 and CD38 co-expression patterns identified transitional B cells (D) and mature (E). Samples with insufficient cell numbers were excluded from the graph (D and F). Statistical differences between RA and SLE patients and normal donors were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

BAFF Receptor Expression Negatively Correlates with Disease Activity in SLE Patients

Previous reports have shown that CD19+ B cells negatively correlated with disease activity in SLE patients as determined by SLEDAI scores (31). The disease activity score for the SLE patients in this study are shown in Table 3. We did not observe a difference in SLE patient BAFF-R expression from our normal donors (Figure 16A), Our studies confirm previous reports, and show that BAFF-R MFI on total CD19+ B cells negatively correlates ($r=-0.6874$ and $p=0.0250$) with SLEDAI scores from our SLE patient cohort (see Figure 17). In addition, BAFF-R MFI was also shown to negatively correlate ($r=-0.4304$ and $p=0.0423$) with BAFF MFI on CD19+ B cells from RA patients (data not shown).

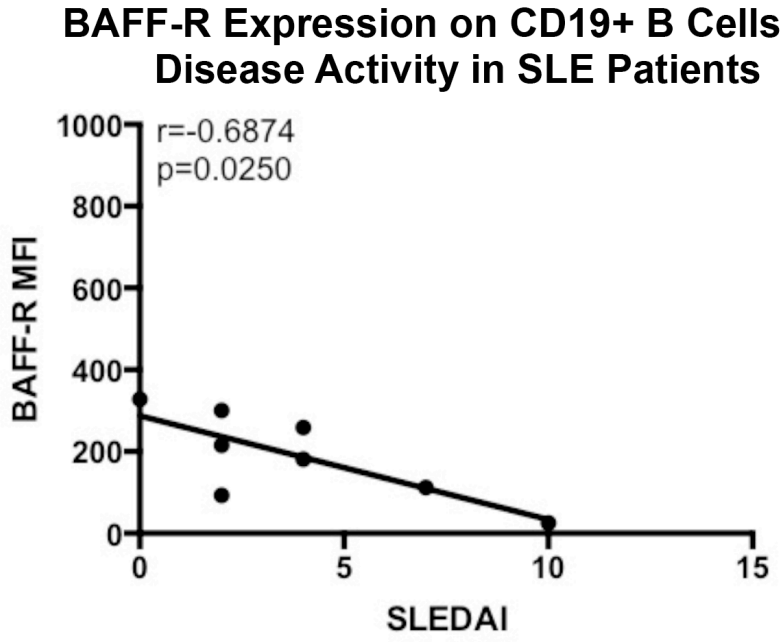

Figure 17: BAFF-R Correlates with Disease Activity in SLE Patients. MFI values for BAFF-R expression on CD19+ B cells from SLE patients were determined and graphed in Figure 15A. The Spearman's rho test indicates that BAFF-R expression on CD19+ B cells is negatively correlated with disease activity as determined by SLEDAI in n=8 SLE patients.

TACI Expression is Elevated on B Cells Subsets in RA and SLE Patients

TACI receptor has been shown to be elevated on SLE patients with active disease and the highest levels are seen on SLE patients with lupus nephritis (31). Living CD19+ B cells were gated in normal, RA, and SLE patient samples as shown in Figure 12. As seen in Figure 18A, no difference in TACI expression on total CD19+ B cells. In both RA and SLE patients, TACI is elevated on IgM+ B cells (Figure 16B), IgM+CD27+ memory B cells (Figure 18C), transitional (Figure 18D), and mature B cells (Figure 18E) compared to healthy donors. Surface APRIL expression also positively correlated with TACI receptor expression on the same CD19+ B cells from RA patients (data not shown).

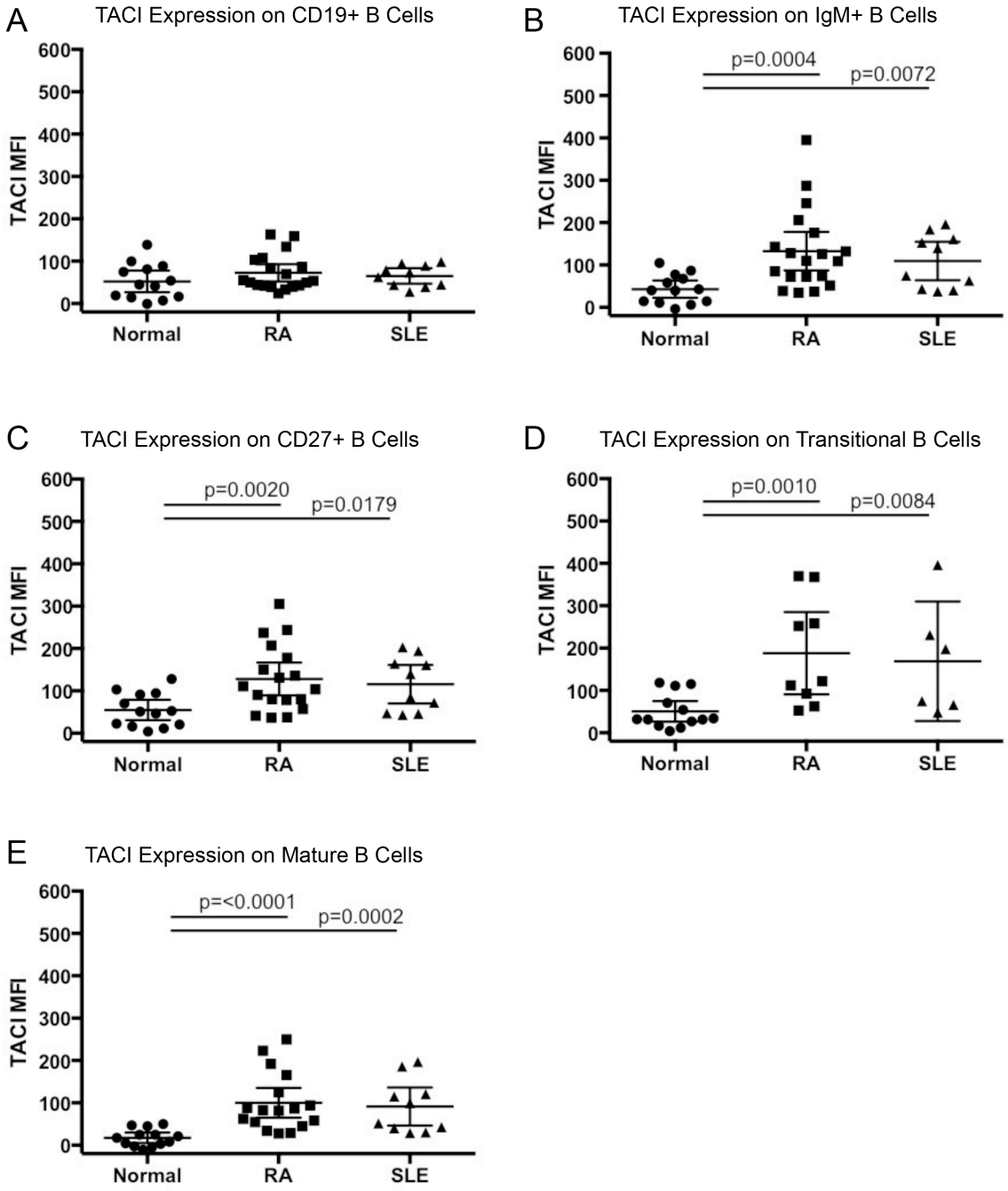

Figure 18. **TACI Receptor is Elevated on IgM+ B cells from RA and SLE Patients.** Graphed is the median fluorescence intensity (MFI) of TACI staining on CD19+ B cells (A), CD19+IgM+ B cells (B), and CD19+IgM+27+ memory B cells (C). TACI expression on CD19+IgM+CD27- B cells using CD24 and CD38 co-expression patterns identified transitional B cells (D) and mature (E). Samples with insufficient cell numbers were excluded from the graph (D and F). Statistical differences between RA and SLE patients and normal donors were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

Table 2: RA Patient Characteristics

Sex (M/F	Age (yrs.)	Disease Duration (yrs)	DAS28 Score	Rheumatoid Factor	Treatment
F	70	4	4.73	neg	MTX, HCQ
F	61	6	4.99	pos	ADA, MTX
M	46	1	3.7	pos	LEF
F	56	28	ND	pos	MTX, PRED
F	21	3	4.35	pos	NONE
F	42	11	2.35	pos	Etanercept
F	68	10	ND	ND	MTX
F	66	24	1.56	pos	LEF
F	64	5	2.35	pos	MTX, HCQ
F	56	29	5.13	pos	MTX, PRED, LEF
M	60	4	6.95	pos	NONE
F	57	15	1.53	pos	MTX, Etanercept
F	33	1	4.58	neg	HCQ
F	38	9	2.3	neg	MTX, Rituximab
F	64	6	2.8	neg	MTX, HCQ
F	62	1	2.72	pos	MTX ,PDN
F	75	15	3.57	ND	MTX, Infliximab
F	75	5	4.75	pos	MTX

NR: not reported, MTX: methotrexate, PRED: prednisone, LEF: leflunomide, HCQ: hydroxychloroquine, ADA: Adalimumab

Table 3: SLE Patient Characteristics

Sex (M/F)	Age (yrs.)	Disease Duration (yrs)	SLEDAI	Treatment	Clinical Features
F	43	3	10	AZA, HCQ	ANA, smith, anemia, thrombocytopenia
F	48	35	2	MMF	seizures, nephritis, rash, ANA
F	35	9	NR	HCQ	ANA dsDNA, nephritis, arthritis, serositis
F	32	10	NR	HCQ	nephritis, ANA
F	24	8	2	MMF, PRED, HCQ	ANA dsDNA, rash, arthritis
F	59	10	0	HCQ	rash, alopecia, arthritis
F	34	1	4	None	alopecia, arthritis
F	26	2	7	PDN	rash, oral ulcers, arthritis
F	48	2	4	HCQ, PDN	arthritis, rash, alopecia
F	64	4	2	AZA	alopecia, arthritis

NR: not reported, PRED: prednisone, AZA: azathioprine, HCQ: hydroxychloroquine, MMF: mycophenolate mofetil

Discussion

B cells have multiple roles in the pathogenesis or RA and SLE. The primary pathogenic function is the production and secretion of autoantibodies. B cell also serve as antigen-presenting cells (APCs) where they can activate T cells and secrete pro-inflammatory cytokines (32, 33). B cell disturbances exist in both RA and SLE. Increased numbers of transitional, naïve mature, and CD27+ memory B cells have been measured in SLE patients (34). BAFF and APRIL and their receptors play important roles in these B cell mediated immune responses.

The majority of functional studies of APRIL, BAFF and their receptors have been focused on the role of the soluble forms of APRIL and BAFF in the serum and synovial fluid. Recently surface forms of these molecules have been identified. Surface BAFF is expressed on leukemic B cells, and on naïve, memory, and plasma B cells from SLE patients with severe disease activity (18, 19). Surface forms of APRIL have been identified on cell lines from lymphoid and myeloid malignancies as well as macrophages in the synovial tissue of RA patients (19, 20, 22). Therefore, we sought to determine whether B cells and B cell subsets in RA and SLE PB express surface APRIL and BAFF. We show that RA and SLE patients have elevated surface APRIL and BAFF expression on total CD19+ and developmental B cell subsets (Figure 13 and 4). In addition, we show that surface APRIL expression on total CD19+ B cells positively correlates with disease activity in our RA patient cohort (Figure 14).

Alterations in BAFF-R, which binds BAFF, and in TACI that binds both APRIL and BAFF have been reported in SLE patients. Previous studies report reduced BAFF-R expression on SLE B cells, which is negatively correlated with disease activity in SLE patients (31) and functional studies have shown that BAFF-R is decreased when exposed

to high levels of soluble BAFF (35). This phenomenon may explain low levels of BAFF-R staining shown in our SLE patient samples (Figure 16). However, RA patients had elevated levels of BAFF-R expression on IgM+, memory, transitional, and mature B cell subsets (Figure 16). We confirm previous reports that BAFF-R expression on CD19+ B cells negatively correlates with disease activity in SLE patients (Figure 17). Increased TACI receptor expression has been reported in patients with active SLE and with lupus nephritis (31). TACI is elevated on IgM+, memory, transitional, and mature B cell subsets (Figure 18)

Alternative APRIL splice forms give rise to surface APRIL (36). BAFF is known to be cleaved at the cell surface; therefore, surface BAFF is a naturally occurring form of this protein (36). In contrast, APRIL is processed intracellularly within the Golgi apparatus and is then secreted from the cell (37). An intergenic splice form exists that is generated from the combination of exons 1-6 of TWEAK (a closely related TNF family member) and exons 2-6 of APRIL producing TWE-PRIL (38). TWE-PRIL has the transmembrane domain of TWEAK and the trimeric form of APRIL as an uncleaveable membrane bound protein (38). TWE-PRIL as well as alternative splice forms of APRIL may lead to the surface expression of APRIL seen in Figure 14 (36). The cells that express surface APRIL and its function in the pathology of rheumatic diseases has not been elucidated. In addition, it is not known whether the soluble and surface forms of APRIL have similar functions. The surface expression of APRIL in malignant cell lines suggests that this surface expression may be aberrant. Current work in our lab is investigating potential splice variants of APRIL not found in PBMNCs from normal donors.

The presence of elevated levels of APRIL and BAFF in the serum and synovial fluid of RA and SLE patients implicated these molecules as upstream mediators of B cell autoimmunity and led to the development of biologics to target these pathways (15, 16, 28, 31). Recently, the FDA approved Belimumab for the treatment of autoantibody positive SLE for patients who do not respond to the standard of care therapy. Belimumab blocks BAFF from binding its receptors BAFF-R and TACI (39). The implication of APRIL in B-cell mediated autoimmune diseases led to the recent development of Atacicept, a humanized fusion protein of the Fc portion of IgG and TACI. Atacicept antagonizes APRIL as well as another cytokine, BAFF and is currently in Phase III clinical trials for the treatment of SLE (40). Since APRIL and BAFF have overlapping effects in B cell survival and maintenance, this drug provides promise in targeting multiple autoreactive B cell subsets including plasma cells. Atacicept may prove more effective in treating RA as it targets upstream inflammatory initiating events instead of blocking cytokines such as TNF-α. While these new drugs act on upstream inflammatory initiating events, determination of BAFF and APRIL levels are not a part of patient selection criteria for their administration, despite the diverse nature of these diseases.

In conclusion, APRIL is expressed on total CD19+ and developmental B cell subsets in RA and SLE patients and positively correlates with DAS28 measurement of disease activity in RA patients. In addition, surface BAFF is elevated on CD19+ and developmental B cell subsets in RA and SLE patients with highest expression found on CD27+ B cells in RA patients. BAFF-R is elevated on B cell subsets in RA patients and negatively correlates with disease activity in SLE patients. In both RA and SLE, TACI receptor was elevated on B cell subsets with the highest expression seen on transitional B

cells. These results show that in SLE and in RA the patient's B cells themselves serve as a reservoir of surface APRIL and BAFF that provides a potential source of stimulation for the BAFF-R and TACI receptors that are also upregulated on these cells. Elevated expression of BAFF-R and TACI on transitional B cells suggests that these cells may be more responsive to APRIL and BAFF signaling. More studies are needed to determine the function of surface APRIL and BAFF in the B cell signaling pathways in RA and SLE.

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CHAPTER FOUR

THYMIC STROMAL LYMPHOPOIETIN (TSLP) IS ELEVATED IN THE PLASMA OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND IN THE SYNOVIAL FLUID OF RHEUMATOID ARTHRITIS PATIENTS

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Abstract

Introduction: Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine that is produced by epithelial and stromal cells and is important in T and B cell development. TSLP has been associated with the pathogenesis of airway diseases such as asthma and is thought to cause the progression of cancer. Recent studies suggest that TSLP may also play a role in inflammatory arthritis by activating dendritic cells, which will ultimately lead to the activation of native T-cells and their differentiation into T help 2 (Th2) cells and the production of inflammatory cytokines such as TNF-a. In this study we evaluated TSLP in systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA), which are inflammatory B-cell mediated autoimmune diseases while osteoarthritis is considered to be non-inflammatory. By ELISA, we found elevated levels of TSLP in plasma from SLE patients and in synovial fluid of RA patients. The aim of this study is to compare of soluble TSLP from SLE and RA patients to non-inflammatory OA patients and determine the role of TSLP in RA and SLE.

Methods: Peripheral blood (PB) and synovial fluid (SF) were collected from OA, RA, and SLE patients through an IRB-approved protocol. All patients were receiving treatment during the study. The TSLP levels in plasma and SF from patient samples were measured by ELISA using the Human TSLP ELISA MAX kit (Biolegend) according to the manufacture's instructions. The minimum detection level of the ELISA was 3.9 pg/mL. The Mann and Whitney one-tailed U test was used to compare the TSLP concentration between patient groups. A *P* value of less than 0.05 was considered significant.

Results: Plasma and SF samples obtained from OA, RA, and SLE patients were analyzed by ELISA. The TSLP levels in RA and SLE plasma and SF were compared to

OA samples. TSLP was elevated in plasma from SLE patients compared to OA ($p=0.0276$) and RA ($p=0.007$) plasma. RA SF had higher levels of TSLP than OA SF ($p=0.0101$).

Conclusions: TSLP was elevated in SLE plasma at levels significantly higher than RA and OA plasma. This is the first study to show that TSLP is elevated in SLE patients. RA SF had higher levels of TSLP than OA SF confirming published reports. These data further implicate TSLP in inflammation within B-cell mediated autoimmune diseases. Ongoing studies are being focused on the role of TSLP in inflammation and correlation of TSLP with disease severity and treatment.

Abbreviations

TSLP - Thymic Stromal Lymphopoietin

IL – Interleukin

IL-7Rα – Interleukin Seven Receptor Alpha Subunit

TSLP-R - Thymic Stromal Lymphopoietin Receptor

Jak-STAT - Janus Kinase-Signal Transducer and Activator of Transcription

DC – Dendritic Cell

NK cell – Natural Killer Cell

TLR – Toll-Like Receptor

TNF-α - Tumor necrosis factor alpha

NF-kb – Nuclear Factor Kappa Beta

APC – Antigen Presenting Cells

Th2 - T Helper 2 Cells

RA - Rheumatoid Arthritis

SLE - Systemic Lupus Erythematosus

OA – Osteoarthritis

DAS28 - Disease Activity Score out of 28 Joints

ELISA – Enzyme Linked Immunesorbent Assay

PBMNCs - Peripheral Blood Mononuclear Cells

CD – Cluster of Differentiation

CCLX – Chemokine (C-C motif) Ligand

BAFF – B cell Activating Factor

Introduction

Thymic stromal lymphopoietin (TSLP) is an IL-7 like cytokine that signals through the TSLP receptor (TSLP-R). This receptor consists of an IL-7 receptor alpha (IL-7Rα) subunit and a TSLP-R subunit and upon activation signals through the Jak-STAT pathway in human cells. TSLP is primarily produced by epithelial cells in the skin, gastrointestinal tract, thymus, and lungs and by immune cells such as dendritic cells (DCs), mast cells, basophils, and fibroblasts (1-5). Several immune cells can respond to TSLP induced stimulation since TSLP-R is expressed on DCs, NK cells, mast cells, and T and B cells (6).

TSLP may provide a link between the innate and adaptive immune system. Toll-like receptor (TLR) ligands induce the expression of TSLP in DC and monocytes (1). Upon activation these DCs produce pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 through the activation of NF-κβ pathway. These DCs then act as antigen-presenting cells (APCs) to TSLP responsive immune cells such as CD4+ helper T cells and B cells (7). TSLP acts on DCs to differentiate native CD4+ T-cells into T helper 2 (Th2) cells, which produce pro-inflammatory cytokines such as TNF-α (8-10). TSLP also plays an important in T cell, B cell, and basophil development and homeostasis (11, 12).

Increased levels of TSLP may lead to a breakdown of the normal immune cell homeostasis and contribute to the inflammatory processes in RA and other inflammatory autoimmune diseases. An overview of the cells targeted by TSLP and the inflammatory cytokines produced upon TSLP activation are shown in Figure 19. TSLP has been implicated in the pathogenesis of asthma, atopic dermatitis, systemic sclerosis, atherosclerosis, and cancer (4, 13-15). In the collagen induced arthritis mouse model,

TSLP exacerbated their arthritis and these mice developed characteristics of a RA-like disease. When these mice were treated with anti-TSLP monoclonal antibodies, a reduction in arthritis severity was observed suggesting that TSLP may play a role in the inflammatory arthritis in these animals (16). More recently, TSLP has been implicated in the pathogenesis or RA since it is elevated in patients with RA; however, no association between TSLP and markers of inflammation and disease duration were found (16, 17). In RA patient synovial fluid (SF), TSLP was shown to be co-elevated with TNF-α. Moreover, RA synovial fibroblasts produce TSLP when treated with TNF-α suggesting a role for TSLP in the propagation of RA (16).

Studies with RA patients have shown that TSLP is elevated in autoimmune disease characterized by autoantibody production. Systemic lupus erythematous (SLE) shares similar characteristics with RA, as these patients have numerous autoantibodies and elevated inflammatory cytokine production. In addition, both RA and SLE patients are often treated by the same therapeutics. Therefore, the aim of this study was to compare the level of soluble TSLP from SLE and RA patients to non-inflammatory OA patients, and examine the role of TSLP in RA and SLE. By ELISA, we found elevated levels of TSLP in plasma from SLE patients and in synovial fluid of RA patients.

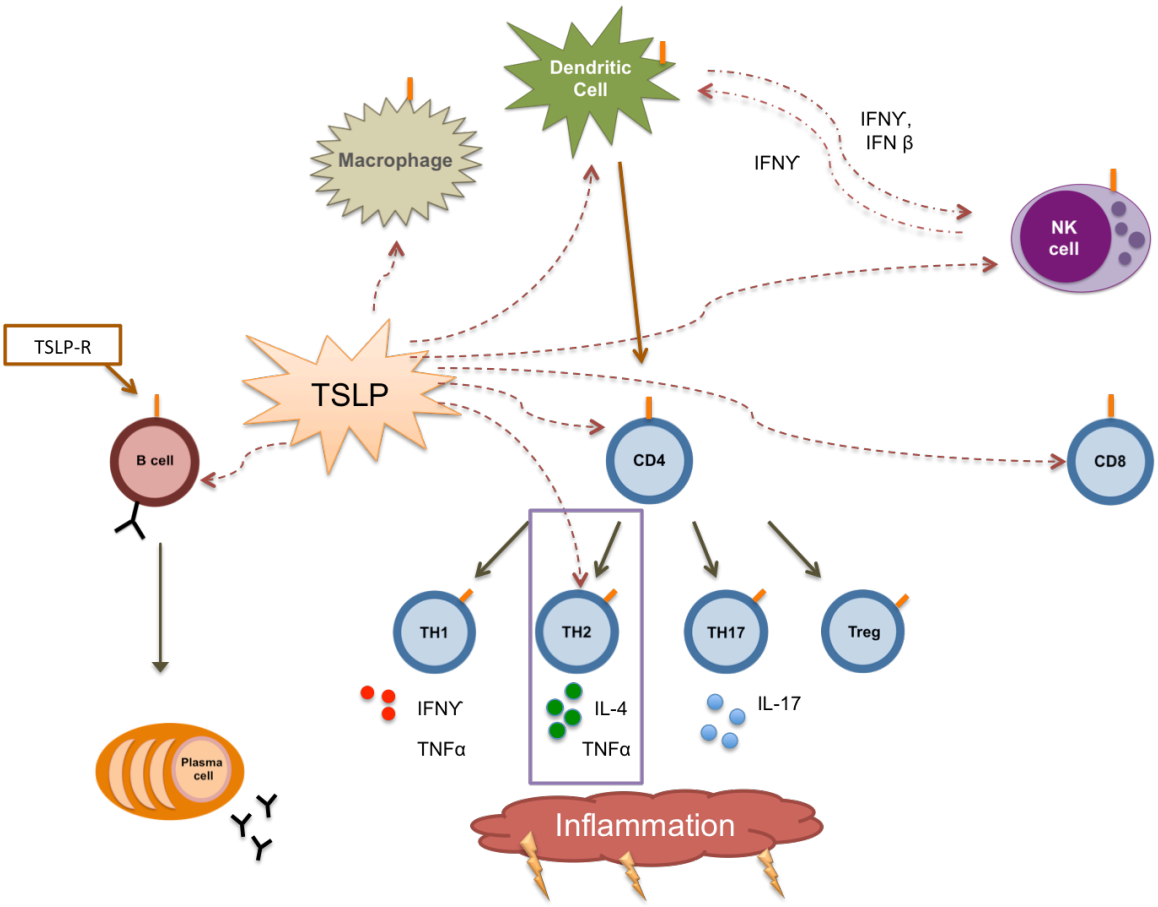

Figure 19. **The Potential Role of TSLP in Inflammation.** The cytokine TSLP is produced by stromal cells, DCs, and fibroblasts cells that line the synovium of joints. The TSLP receptor is expressed on multiple types of immune cells and its effects depend upon which cells it activates. Studies have shown that TSLP exerts most of its effects on the DC interaction with naïve CD4+ T cells. It induces their differentiation into Th2 cells, which produce inflammatory cytokines such as TNF-α. IN general, the effect of TSLP leads to an inflammatory response.

Materials and Methods

Subjects

Subjects were enrolled in this study and gave informed consent under a protocol approved by the Loma Linda University (LLU) Institutional Review Board. All RA, SLE and OA patients were from Loma Linda University Division of Rheumatology Clinic, Loma Linda California or Beaver Medical Group Rheumatology Clinic, Redlands, CA USA. All patients had a clinical diagnosis of RA and met the 1987 American College of Rheumatology criteria (18).

Sample Preparation

Blood samples were collected by venipuncture in tubes containing acid citrate dextrose (ACD) from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Whole blood samples were centrifuged at 1500rpm for 15 minutes to remove plasma. The patient plasma was aliquoted and stored at -20C for future assays. Peripheral blood mononuclear cells (PBMNCs) were then isolated by ammonium chloride lysis of red blood cells in OA, RA and SLE patients and normal PB samples. All PBMNCs were stored in freezing media supplemented with 20% trehalose in liquid nitrogen. Synovial fluid was aspirated from the knee joint of OA and RA patients and stored at -20C for future assays.

Soluble Cytokine Quantification

Levels of TSLP in the plasma of OA, RA and SLE were determined by a sandwich ELISA (Human TSLP ELISA MAX Deluxe Kit, Biolegend San Diego, CA). Synovial fluid TSLP concentrations from OA and RA patients were also assessed by

ELISA without treatment with hyaluronidase. ELISAs were performed according to manufacture's instructions on freshly thawed plasma and synovial fluid. The ELISA plates were read at 450nm absorbance using the uQuant 96 well plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). A standard curve was run in triplicate for each plate and samples were run in triplicate. TSLP plasma levels were calculated using a 4-parameter logistic curve fit model in Bio-Tek software KCJunior V1.6 (Winooski, VT, USA). Samples with a coefficient of variability (%CV) greater than 15% were excluded.

Flow Cytometry

PBMNCs from RA, SLE, and OA donors were stained with mAbs in PBS for 30 minutes in the dark at 4ºC. Cells were then washed with PBS and stained with Fixable Viability Dye eFluor® 450 (eBioscience) to distinguish between living and dead cells and incubated for 30 minutes at 4ºC in the dark. Cells were subsequently washed and fixed with a 1% paraformaldehyde solution before analysis using the MACSQuant® Analyzer (Miltenyi Biotec). The following isotype control antibodies were used: mouse IgG1 FITC, mouse IgG1 PE-Cy5, mouse IgG1 PE–Cy7 (eBioscience, San Diego, CA, USA); mouse IgG1 APC-Cy7, mouse IgG1 PerCP (Biolegend, San Diego, CA, USA); mouse IgG1 PE, mouse IgG1 APC (Miltenyl Biotech Inc., Auburn, CA, USA). The anti-human antibodies used were: CD8 FITC, CD4 PE, CD56 FITC, CD19 FITC, and CD38 PE (BD Biosciences, San Jose, CA, USA) and IgM PE-Cy5, CD24 PE-Cy7, TSLP-R APC, CD27 APC Cy7 (Biolegend, San Diego, CA, USA). Compensation beads (anti-mouse Ig k compensation beads, BD Biosciences, San Jose, CA, USA) and cells stained with Fixable Viability Dye eFluor® 450 were used for machine compensation settings.

Flow cytometry data analysis was performed using Flowjo data analysis software (TreeStar, Ashland, OR).

Statistical Analysis

Statistical differences were determined using non-parametric Mann-Whitney one-tailed tests using GraphPad PRISM software (GraphPad, San Diego, CA, USA). Differences were considered to be statistically significant for $p<0.05$. Grubb's Test was used to exclude significant outliers. The mean and 95% confidence interval is shown in each graph. The p values are provided in each figure.

Results

TSLP is Elevated in the Synovial Fluid of RA Patients

TSLP has been shown to be elevated in the synovial fluid along with the inflammatory cytokine TNF-α and not in the synovial fluid of OA patients (16). More recently, elevated levels of TSLP in RA SF may play a role in activating myeloid DCs (19). Therefore, we sought to determine the levels of TSLP in the synovial fluid in our cohort of RA patients compared to non-inflammatory OA. Synovial fluid from the knee joint of both OA (n=6) and RA (n=13) patients was obtained and assessed by ELISA to determine the concentration of TSLP within the joint. Our results are consistent with previous findings that the concentration of TSLP was significantly increased in the synovial fluid of RA patients compared to OA patients (Figure 20).

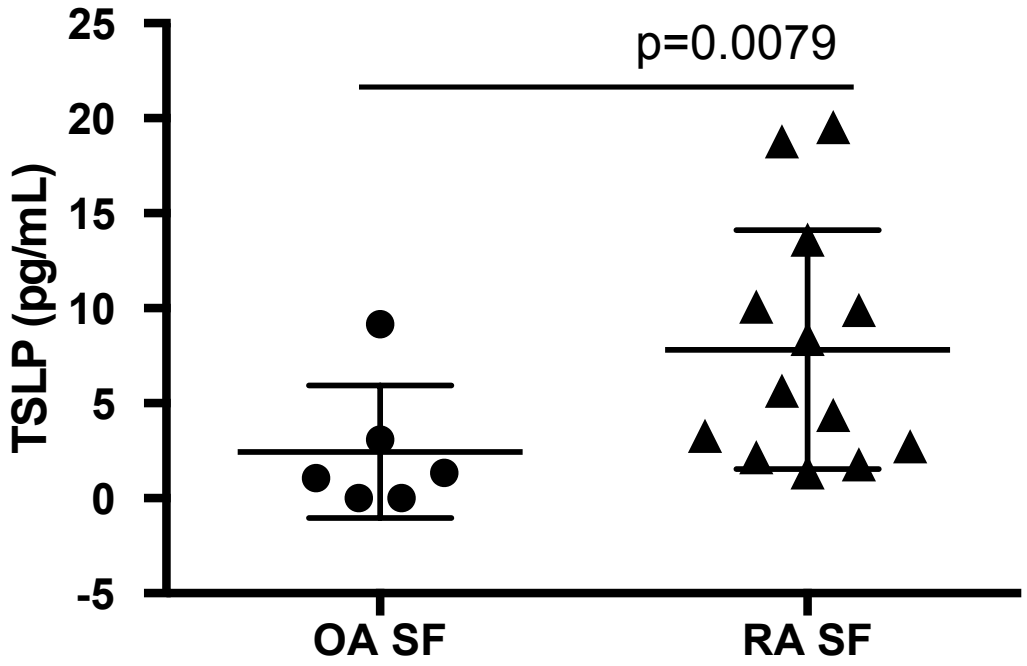

Figure 20. TSLP is Elevated in the Synovial Fluid of RA Patients. The concentration of TSLP in synovial fluid of RA (n=13) and OA (n=6) was determined by ELISA. Statistical differences between RA and OA patients were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

TSLP is Elevated in the Plasma of SLE Patients

TSLP has been shown to be elevated locally in the joints of RA patients (16, 17). Next we sought to determine if TSLP was elevated in systemic autoimmune diseases RA and SLE compared to non-inflammatory OA. The plasma levels of TSLP in RA (n=11), SLE (n=10) and OA (n=5) patients were analyzed by ELISA. TSLP was elevated in the synovial fluid of RA patients (Figure 20), but no difference was observed between plasma levels of TSLP in RA and OA patient samples (Figure 21). However, we found that TSLP was significantly elevated in the plasma of SLE patients (Figure 22). This data suggests that TSLP may act locally in the joints of RA patients and systemically in SLE patients.

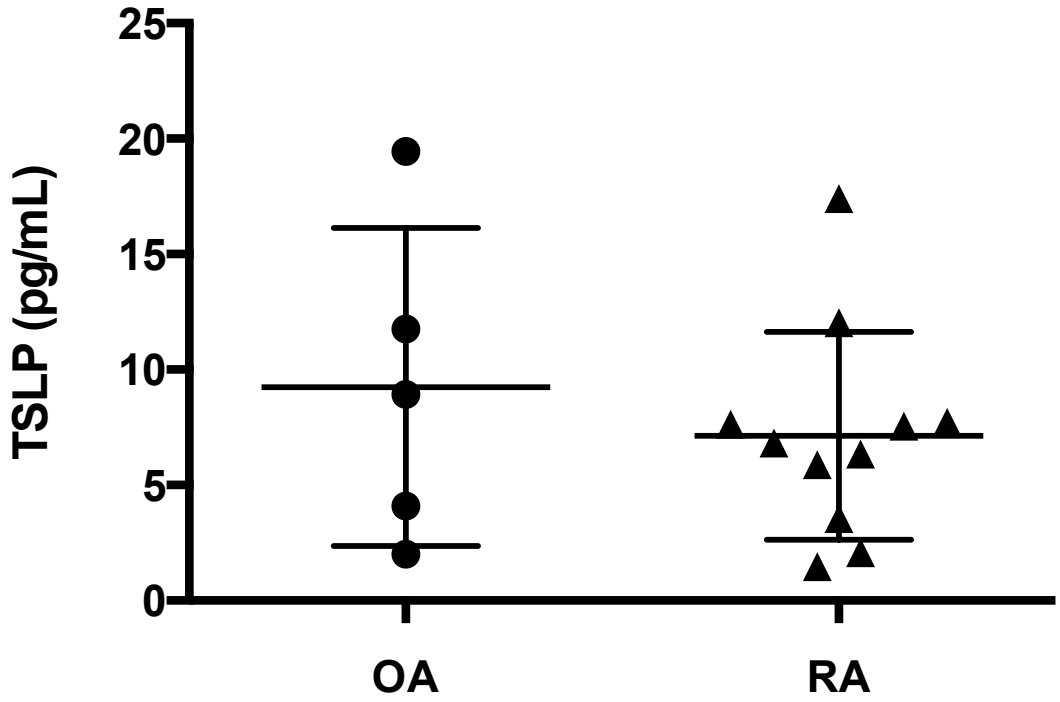

Figure 21. **No Difference in TSLP Levels Were Observed Between RA and OA Patient Plasma.** The concentration of TSLP in plasma of RA (n=11) and OA (n=5) was determined by ELISA. Statistical differences between RA and OA patients were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

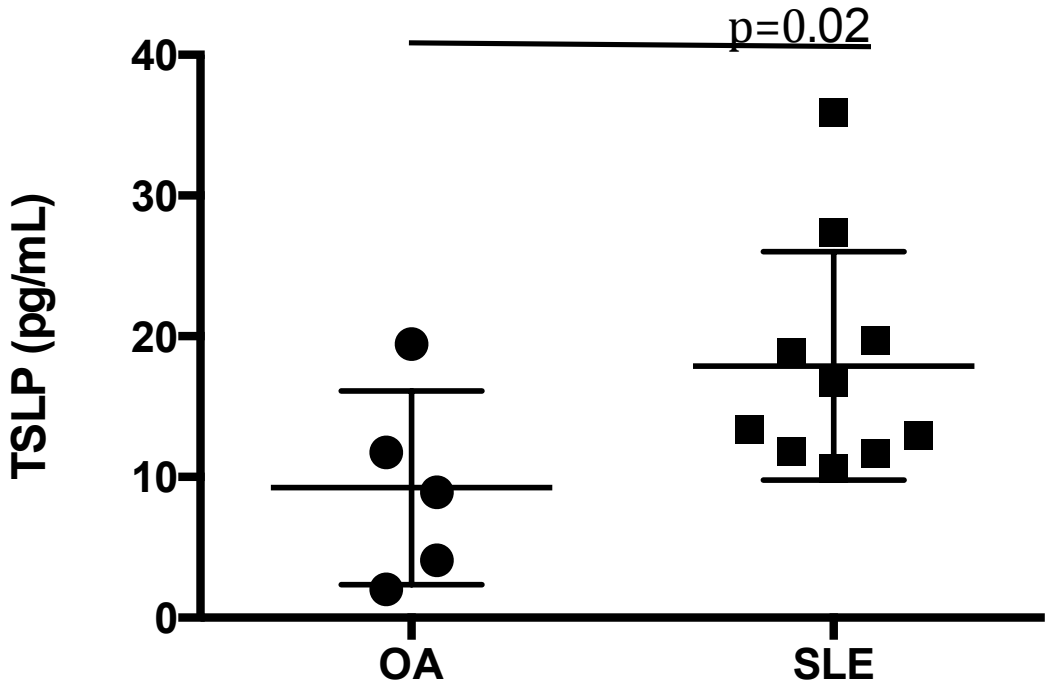

Figure 22. **TSLP is Elevated in SLE Patient Plasma.** The concentration of TSLP in plasma of SLE (n=10) and OA (n=5) was determined by ELISA. Statistical differences between SLE and OA patients were determined by Mann-Whitney one-tailed test and differences were considered to be statistically significant for $p<0.05$.

Cellular Target of Increased TSLP Expression

Next, we sought to determine the cellular target of this increased TSLP production. The TSLP-R expression on several immune cell populations was determined by flow cytometry. Myeloid DCs have been shown to have the high co-expression of TSLP-R and IL-7Rα (20). TSLP-R is also expressed on NK cells, mast cells, and T and B cells (6). Previous reports have shown that TSLP-R is elevated on Th2 cells as well (21). Therefore, we sought to determine the level of TSLP-R expression on these immune cell populations. The following immune cells were identified by cell surface markers: NK cells (CD56), DCs (CD11c), helper T cells (CD3 and CD4), cytotoxic T cells (CD3 and CD8), and immature and mature B cells subsets were identified based on the combination of CD19, CD27, IgM, CD38, and CD24. Preliminary data from 2 independent experiments suggests that mature B cells (CD19+CD27-IgM+) from RA patients (n=3) have elevated levels of TSLP-R expression compared to normal donors. We did not observe a difference between TSLP-R expression in T cell subsets, NK cells, and DCs within the PBMNCs of RA and SLE patients and normal donors.

Discussion

The role of TSLP in inflammation is an area of emerging investigation. RA and SLE are B cell mediated systemic autoimmune disease but also involve many other immune cells that participate in the inflammatory processes that lead to the homing of immune cells to the joint and the damage to the joint caused by these cells. While the exact pathways that lead to the development of RA and SLE are not known, genetic risk factors are known. Current theories hypothesize that these RA and SLE patients have a genetic risk of developing these diseases and when they encounter an environmental

exposure causing inflammation this leads to tissue damage and propagation of the disease by many immune cells (2-5). TSLP may be a link between the normal maintenance of immune cells as well as their activation by environmental triggers through TLRs.

Initiation of the pro-inflammatory effects of TSLP may be due to an environmental trigger such as a bacterial or viral infection through the involvement of TLRs in TSLP production. TSLP expression in mouse DCs and human monocytes is induced by TLR ligands zymosan (TLR2 ligand), LPS (TLR4 ligand), and CpG (TLR9 ligand), which in turn increased the production of TSLP by DCs (1). TLR2, 3, 4, and 9 ligands have been shown to induce TSLP promoted human DC activation leading to the production of cytokines known to initiate Th2 and Th17 activity in RA joints (1, 10, 19). In addition to Th2 cytokines, DCs produce CCL17 and CCL22 chemokines that induce the migration of immune cells (22). Both CCL17 and CCL22 are elevated in DCs from RA patients and are believed to lead to the homing of immune cells to the RA synovium (23, 24). TSLP production by synovial fibroblast from RA patients was induced by TLR3 and 4 stimulation through a NF-κβ mediated mechanism (25). TLR stimulation of epithelial cells induced class switch recombination in B cells to enhance mucosal immunity. This process is enhanced during TSLP promoted BAFF production by DCs (26). The ability of TSLP to activate DCs and synovial fibroblasts and lead to the production of inflammatory Th2 and Th17 cells and influence B cell antibody production suggests that this cytokine may provide a attractive target for therapeutics.

TSLP may also serve as a chemokine as it can promote the migration of DC's to areas of inflammation by serving as a messenger between the epithelia and innate immune cells (27). Viral RNAs induce the production of TSLP in epithelial cells, a

major producer of TSLP (28). DCs treated with TSLP upregulate survival proteins, MHC class II and other co-stimulatory molecules enhancing their ability to interact and activate other immune cells involved in the pathogenesis of RA and SLE (2, 20). DCs from the synovium of RA patients show increased levels of co-stimulatory molecules with TSLP stimulation (7, 19). IL-32, a cytokine produced by upon cells during bacterial and viral infections, has been shown to be elevated in the synovium of RA patients. Recently, IL-32 was shown to increase TSLP production in monocytes as well as their differentiation into macrophages, which are known to contribute to RA pathogenesis (29).

TSLP supports the survival and differentiation of immune cells. TSLP promotes the development of basophils from bone marrow precursors (12). Murine immature IgM+ B cell development in bone marrow and fetal liver is supported by TSLP (30). TSLP plays a role in helper T cell survival and proliferation and the production of IL-4, IL-5 and IL-13 (21). In the mouse, Th2 cells have higher levels of TSLP-R (21).

Elevated levels of TSLP have been examined in RA, however no studies have expanded to include similar systemic autoimmune conditions such as SLE. TSLP the synovial fluid of RA joints showed co-elevation with a known inflammatory cytokine TNF-α differing from another study that showed no correlation between synovial levels of TSLP and TNF-α (16, 19). In our study, we show that TSLP is elevated in the synovial fluid of RA but not OA patients (Figure 20). In addition, we are the first to show that TSLP is elevated in the plasma of SLE patient when compared to RA and OA patients (Figure 21-4). These data suggest that TSLP may act locally in the joints of RA patients and systemically in SLE patients. Ongoing studies will explore the role of systemically elevated TSLP in the pathogenesis of SLE.

Therapeutics that are currently being used to treat patients with RA and SLE have an impact of the levels of TSLP. Glucocorticoids, immune suppressives often used to treat systemic autoimmune disorders, have been shown to inhibit TSLP potentially through blocking the NF-κβ activation (31). In RA mouse models, treatment with anti-TSLP monoclonal antibodies significantly reduced the arthritis score in these animals (16). In RA patients, TSLP levels were significantly lower in patients that were treated with DMARDS and TNF-α inhibitors (19). Therefore, targeting TLSP either directly with monoclonal antibodies or indirectly by preventing NF-κβ activation through the use of glucocorticoids may prove beneficial in treating the inflammatory events in asthma, dermatitis, arthritis, and lupus.

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CHAPTER FIVE

DISCUSSION

The success of biological therapies in the treatment of RA has dramatically improved the management of this disease and other autoimmune disorders. Newly developed biologics have the potential to selectively target immune components involved in autoimmune disease. The TNF antagonists are an example of targeted therapy and have shown great promise in the treatment of RA and SLE. However, due to the heterogeneous nature of these diseases, the response to these drugs is suboptimal in 30-40% of patients (1-5). Therefore, identifying subsets of patients based on their surface and soluble biomarkers and identifying which therapeutics that will provide the most benefit for these patient cohorts has the potential to rationalize the treatment of RA and SLE patients.

Identification of Patient Cohorts and Therapeutic Response through Genome Wide Association Studies

RA is a complex and heterogeneous disease that has contributions from lymphoid, myeloid and fibroblast cells with in the joint. The use of genome-wide expression analysis (GWAS) has recently been used to identify to major forms of RA—lymphoid and myeloid that contribute to the inflammation in the RA synovium (6). In addition, GWAS studies have identified two main pathways in the pathogenesis that involve TLR signaling and type I interferon signaling (7). Pharmacogenetic studies have great potential to assist in predicting which therapies would be more effective in each type of RA and SLE. This type of big data approach may be the future of personalized medicine in which

your genetic profile will be used to determine genetic susceptibility to disease and therapeutic effectiveness.

TSLP and APRIL Induced IgA Antibody Production by Mucosal B Cells

Intestinal epithelial cells (IECs) play a role in mucosal immunity by secreting TSLP that can signal through TSLP-R on mDCs in the intestine. These TSLP induced DCs promote the differentiation of CD4+ Th cells into Th2 effect T cells (8). These TSLP DCs produce BAFF and APRIL and TLR5 induced IECs produced APRIL, which bind the TACI receptor on mucosal B cells resulting in T cell independent class switch recombination to produce IgA antibodies (9, 10). This provides a link between the microbiome and mucosal immunity.

Viruses as the Environmental Trigger in RA and SLE

Viral infections may serve as environmental triggers for the development of autoimmune disease. Type I interferon is produced by numerous immune cells as a response to viral infections. The combination of type I interferon and immune complex ligation of TLR7 or 9 can lead to the activation of pDCs. Activated DCs present self-antigens to B cells leading to their differentiation to plasma cells (11). Viruses can also induce TLR mediated epithelial cell production of BAFF (12). The increased levels of BAFF may allow autoreactive B cells to escape negative selection resulting in circulating autoreactive B cells. BAFF can also augment the APRIL induced IgA antibody production (10). Non-classical monocytes patrol the vasculature respond to viruses through TLR7 and 8 and produce TNF-α and IL-1β (13). The collaboration of these

immune cells and cytokines implicate a viral environmental trigger in RA and SLE patients with elevated levels of APRIL, BAFF and TSLP.

Identification of RA and SLE Biomarkers and Determination of Their Relationship to Disease Activity

The aim of our studies was to identify easily detectable biomarkers in RA and SLE patients and provide insight into the role of these biomarkers in the pathology of B cell mediated autoimmune diseases.

Surface APRIL as a Biomarker in RA

The cytokine APRIL has been implicated as a potential disease mediator in B cell mediated autoimmune diseases (14). APRIL is elevated in the serum and synovial fluid of RA patients (15, 16). While multiple immune cells secrete APRIL, it is also expressed on the surface of myeloid leukemia cell lines and macrophages in RA synovium (17-22). The expression of surface APRIL by monocyte subsets in normal individuals and its relationship to RA are unknown. In this study, we sought to compare surface APRIL expression on circulating myeloid cells in both normal and autoimmune patients, and to determine whether expression of surface APRIL is related to plasma levels of soluble APRIL and disease activity in RA patients.

Our studies are the first to show that surface APRIL is expressed on circulating myeloid cells in RA patients (Figure 5) and that levels of surface APRIL are highly correlated with disease activity (Figure 7). Monocytes can be divided into 3 distinct subsets based on the co-expression of CD14 and CD16 (23). Pro-inflammatory non-classical monocytes have previously been shown to be elevated in RA patients with

active disease (13, 24). We show that both non-classical (CD14loCD16+) and intermediate (CD14+CD16+) monocyte subsets are increased in RA patients (Fig. 8B) indicating the pool of monocytes in RA patients is skewed toward pro-inflammatory monocyte subsets. Examination of surface APRIL expression on circulating monocyte subsets showed low levels of surface APRIL on intermediate monocytes, and high levels of surface APRIL on non-classical monocytes in normal donors (Fig. 9D). In contrast, surface APRIL was expressed at high levels on all monocyte subsets in RA patients (Fig. 9E). Our findings suggest that surface APRIL could provide an easily detectable biomarker and be a useful selection criterion for the administration of Atacicept. Surface APRIL expression, together with monocyte subset skewing, might provide a prognostic indicator of RA patient response to Atacicept or other drugs that target APRIL (25).

The Potential for the Use of Surface Expression of APRIL, BAFF and Their Receptors as Biomarkers in RA and SLE

APRIL and BAFF and their receptor TACI are elevated in early RA. This suggests that using the combined expression of APRIL, BAFF and the receptors BAFF-R and TACI (seen in Figure 4) could provide a biomarker panel for the diagnosis of RA and SLE patients. In addition, the expression patterns of APRIL and BAFF could provide a rationale for the use of Belimumab and Atacicept in RA and SLE patients. Flow cytometry was used to assess the surface expression of APRIL, BAFF, BAFF-R and TACI on total CD19+ B cells and B cell subsets, and if this surface expression correlated with disease activity in established RA and SLE patients.

Surface APRIL was expressed on total CD19+ and developmental B cell subsets in RA and SLE patients and positively correlated with disease activity in RA patients

(Figure 13-14). Surface BAFF was elevated on CD19+ and developmental B cell subsets in RA and SLE patients with the highest expression found on CD27+ memory B cells in RA patients (Figure 15). BAFF-R was elevated on B cell subsets in RA patients and negatively correlated with disease activity in SLE patients (Figure 16-17). In both RA and SLE, TACI receptor was elevated on B cell subsets with the highest expression identified on transitional B cells (Figure 18). The elevated expression of surface APRIL and BAFF on B cell subsets in established RA and SLE patients suggest that these cytokines may serve as potential biomarkers for the identification of RA and SLE. Further testing is needed to determine how patients with elevated levels of surface APRIL and BAFF respond to targeted therapy by Belimumab and Atacicept.

The Potential for the Use of TSLP as a Systemic Biomarker in SLE and Local Biomarker in RA Patients

TSLP is an IL-7 like cytokine that is produced by epithelial and stromal cells and is important in T and B cell development. Recently, TSLP has been implicated in the pathogenesis of RA as it is elevated in RA patients and enhances an RA-like phenotype in mouse models of RA (26, 27). Moreover, RA synovial fibroblasts produce TSLP when treated with TNF-α suggesting a role for TSLP in the propagation of RA(26). The aim of our study was to compare the level of soluble TSLP from SLE and RA patients to non-inflammatory OA patients, and examine the role of TSLP in RA and SLE. TSLP was elevated in the synovial fluid of RA but not OA patients (Figure 20). In addition, we are the first to show that TSLP is elevated in the plasma of SLE patient when compared to RA and OA patients (Figure 21-22). These data suggest that TSLP may act locally in the joints of RA patients and systemically in SLE patients. Future studies will need to

determine the role of systemically elevated TSLP and the immune cell signaling pathways induced by TSLP in SLE.

Conclusions

The studies described in Chapters 2-4 examine biological relevant biomarkers to identify RA and SLE patients and the potential use of the expression patterns of these cytokines in providing a rationale to guide treatment strategies that target APRIL and BAFF. In addition, our study also implicates the cytokine TSLP in SLE pathogenesis. Targeting this cytokine may be useful in the treatment of RA and SLE patients and therapeutics aimed at targeting TSLP are currently under development (28).

Future Directions

Alternative splicing gives rise to APRIL isoforms that may lead to the surface expression of APRIL. It is not known whether alternative splice or another mechanism results in the surface expression of APRIL. Future studies will identify APRIL isoforms in RA and SLE PBMNCs. Preliminary studies have shown that a subset of our RA and SLE patients express APRIL splice variants. It is also unclear whether surface APRIL can generate pro-survival signals that are produced by soluble APRIL and whether drugs that antagonize APRIL will be effective in patients with elevated surface APRIL expression. More studies are needed to determine the function of surface APRIL and BAFF in the B cell signaling pathways in RA and SLE, and how patients with elevated levels of surface APRIL and BAFF respond to targeted therapy by Belimumab and Atacicept.

TSLP is elevated in the joint of RA patients and systemically in SLE patients. The role of these elevated TSLP levels in immune response in RA and SLE patients is unclear

as studies in asthma show a skewing toward a Th2 response and a Th17 response in RA patients. Future studies will focus on the role of TSLP in inflammation and correlation of TSLP with disease activity in RA and SLE and identify therapeutics that will be effective in treating the subset of patients with elevated TSLP. These studies will provide a rationale for the use of APRIL and TSLP as biomarkers in RA and SLE.

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